FEB 5 1923 Medical Lib

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXVII, No. 2 FEBRUARY 1, 1923

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

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WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

INFLUENZA-LIKE BACILLI ISOLATED FROM CATS.

By T. M. RIVERS, M.D., and STANHOPE BAYNE-JONES, M.D.

(*From the Department of Pathology and Bacteriology of the Johns Hopkins University, Baltimore.*)

(Received for publication, August 15, 1922.)

Influenza-like bacilli isolated from a throat and a lung culture have recently been described (1). Since it was realized that there existed in man certain bacilli which might be mistaken easily for true influenza bacilli, we were interested to know if similar organisms were present also in animals. In examining the cultures from the throats of 50 normal cats during February, 1922, in a search for hemolytic streptococci (2), we were impressed by the number of colonies of Gram-negative bacilli on the rabbit blood agar plates which looked like those of influenza bacilli. Throat cultures from fifteen of the cats were chosen for study and strains of Gram-negative bacilli from seven were considered of enough importance to report.

The old methods of testing the growth requirements of influenza bacilli were inadequate and must have led to many mistakes. Although knowledge of the growth accessory factors of these bacilli is not complete at present, still an advance has been made in that it is now known that two accessory substances are essential (3–8). By using these substances separately it has been possible to show that certain bacilli similar to influenza bacilli are distinctly different from them (1, 9). The preparation of the growth accessory substances (9), of a medium for demonstrating sugar fermentations (10), and of a clear liquid medium suitable for influenza bacilli (10) has been described already but will be repeated here for convenience and clarity of presentation.

The growth accessory substances were prepared in the following manner.

(*a*) *Autoclave-Labile Factor.*—A pure culture of brewers' yeast was grown on malt agar, pH 5 to 6, scraped off, and washed in physiological salt solution three

times. The yeast cells were added to five or ten volumes of distilled water, brought to a boil, allowed to stand on ice over night, filtered through paper, and sterilized by means of a Mandler filter.

(*b*) *Autoclave-Stable Factor* (*Hematin*).—Red blood cells from 100 cc. of blood were washed three times in physiological salt solution, laked with distilled water, and precipitated with 95 per cent alcohol. The precipitate was boiled with weak acid alcohol (H_2SO_4) until colorless and then filtered. The filtrate was partially saturated with NaCl and filtered. The precipitate was washed thoroughly on the filter with distilled water, dissolved in warm acid alcohol, reprecipitated with NaCl, filtered, washed, taken up in acid alcohol, made slightly alkaline (pH 7.4) with NaOH, and brought up to a volume of 100 cc. with 95 per cent alcohol. While hematin prepared in this way may not be absolutely pure, it is relatively so and answers the purpose desired in this work.

The autoclave-stable substance was added to the different media in quantities of 2 cc. per 100 cc. and was always autoclaved. The autoclave-labile factor, 15 cc. per 100 cc., was added to the various media cold or after they had cooled down to 50°C.

Solid Medium for Sugar Fermentations.—

Peptone (Fairchild's)	20 gm.
Sodium chloride	5 gm.
Agar-agar	15 gm.
Distilled water	1,000 cc.

This was boiled, the reaction was adjusted to pH 7.4, and the material was filtered and autoclaved in 100 cc. quantities. After it was taken from the autoclave and while it was still at 95°C., 1 to 2 cc. of washed red blood cells, 10 cc. of a 10 per cent solution of the required sugar, and enough saturated alcoholic solution of brom-cresol purple to give a good color, were added. While still warm the medium was tubed and later incubated for sterility. The coagulated blood cells, it is true, give the medium a dirty appearance but do not interfere with the results.

Hemo-Peptone Water.—

Peptone (Fairchild's)	20 gm.
Sodium chloride	5 gm.
Distilled water	1,000 cc.

Boiled and reaction adjusted to pH 7.4.
10 to 20 cc. of washed red blood cells added and heated to 95°C.
Filtered through paper.
Sterilized through a Mandler filter.
Tubed or flasked in required amounts and incubated for sterility.

This medium is perfectly clear, contains both growth accessory factors, and supports a splendid growth of influenza bacilli.

Description of the Growth, Cultural Reactions, and Growth Requirements of the Bacilli Isolated from Cats.

For convenience the strains will be designated as Nos. C2, C4, C5, C6, C7, C8, and C11.

Growth on 2 Per Cent Rabbit Blood Agar.—Young and old colonies of all seven strains, except Nos. C8 and C11, look exactly like those of true influenza bacilli. Young colonies of Nos. C8 and C11 also look like those of *B. influenzæ*, but old colonies of No. C8 become slightly crinkled, while those of No. C11 become more opaque than real influenza bacilli. All except No. C11 darken the medium similarly to *B. influenzæ*. Plates inoculated with No. C11 after 48 hours begin to show a diffuse hemolysis, somewhat like that caused by *B. coli*. These bacilli grow well under aerobic conditions.

Staining, Morphology, and Motility.—All the strains are distinctly Gram-negative, non-motile, pleomorphic bacilli. Morphologically they cannot be differentiated from influenza bacilli. They do not form spores.

Production of Indole.—Nos. C2, C4, C7, and C11 form indole; Nos. C5, C6, and C8 do not.

Reduction of Nitrates.—All seven strains reduce nitrates to nitrites.

Production of Amylase.—A starch-splitting ferment is not formed by any of the strains.

Hemolysis.—Six of the bacilli cause no hemolysis on blood agar plates. No. C11, however, produces a diffuse hemolysis of the whole plate after 48 hours, similar to that caused by *B. coli*.

Action on Blood-Milk.—To autoclaved milk were added 1 to 2 per cent rabbit blood and enough brom-cresol purple to give a good color. After inoculation the tubes were incubated 2 weeks. No definite change occurred in any of the tubes except the one inoculated with No. C11 which showed acid formation without coagulation.

Fermentation of Sugars.—Dextrose, maltose, mannitol, lactose, sucrose, and xylose were used in testing the power of these bacilli to ferment sugars. Nos. C2, C4, C5, C6, and C7 produced neither acid nor gas from the above sugars. No. C8 fermented dextrose, maltose, and sucrose with the production of acid. Dextrose definitely inhibited the growth of No. C8 both on solid and in liquid media when used in amounts of 1 per cent or more. Growth and acid production occurred, however, when the sugar was used in 0.5 per cent concentration. No. C11 fermented dextrose and lactose with the formation of acid.

In Table I the important characteristics of the seven strains of influenza-like bacilli from cats are summarized.

Growth Requirements.—These bacilli had been cultivated 4 months on artificial media before their growth requirements were studied. None of them grew for more than one to two generations in peptone

water, peptone water and hematin, peptone agar and hematin, meat infusion broth, meat infusion broth and ascitic fluid, meat infusion agar and ascitic fluid, or peptone water and autoclaved yeast extract. None gave a visible growth on meat infusion agar after the first generation and only Nos. C4, C6, and C7 had viable organisms after five daily transplants. These three strains, in spite of no visible growth, continued to have viable organisms up to the tenth transplant, when the experiment was discontinued. All strains, however, grew well in hemo-peptone water and all of them, with the exception of

TABLE I.

Characteristics of the Bacilli Isolated from Cats.

Strain No.	Staining.	Motility.	Reduction of nitrates.	Formation of indole.	Action on blood-milk.	Fermentation of sugars.					
						Dextrose.	Maltose.	Mannitol.	Lactose.	Sucrose.	Xylose.
C2	Gram-negative.	Non-motile.	+	+	Indefinite.	−	−	−	−	−	−
C4	"	"	+	+	"	−	−	−	−	−	−
C5	"	"	+	−	"	−	−	−	−	−	−
C6	"	"	+	−	"	−	−	−	−	−	−
C7	"	"	+	+	"	−	−	−	−	−	−
C8	"	"	+	−	"	+	+	−	−	+	−
C11	"	"	+	+	Acid without coagulation.	+	−	−	+	−	−

Under nitrates, + indicates reduction; under indole, + indicates indole formation, − indicates lack of indole formation; under the sugars, − indicates that no evidence of fermentation was detected, + indicates that fermentation was apparent by the formation of acid without gas.

No. C11, grew profusely in peptone water and filter-sterilized yeast extract. Immediately it is evident that six of the strains (No. C11 excepted) required the addition of only the autoclave-labile factor as an accessory growth substance. In this respect they are similar to the influenza-like bacilli (1) occurring in man. No. C11 is distinctly different from the others in that growth was only obtained in media which contained unautoclaved blood or blood extract. It would not grow even on a medium which supports a good growth of influenza bacilli,—peptone water, hematin, and filter-sterilized yeast extract.

In Tables II and III are summarized the results of testing the growth requirements of the bacilli.

From Table II it is evident that the bacilli studied, except *Bacillus influenzæ* and No. C11, require for their growth only the addition of

TABLE II.

Growth Requirements of the Bacilli Isolated from Cats.

Strain No.	Peptone water.	Peptone water and hematin.	Peptone water and autoclaved yeast extract.	Peptone water and unautoclaved yeast extract.	Hemo-peptone water.	Meat infusion broth.	Peptone agar and hematin.	Meat infusion agar.	Meat infusion agar and ascitic fluid.	Meat infusion broth and ascitic fluid.
B. influenzæ.	−	−	−	−	+	−	−	−	−	−
C2	−	−	−	+	+	−	−	−	−	−
C4	−	−	−	+	+	−	−	?	−	−
C5	−	−	−	+	+	−	−	−	−	−
C6	−	−	−	+	+	−	−	?	−	−
C7	−	−	−	+	+	−	−	?	−	−
C8	−	−	−	+	+	−	−	−	−	−
C11	−	−	−	−	+	−	−	−	−	−

− indicates that successful transplants were made for one to two generations only; ? indicates no visible growth but viable organisms after many transplants; + indicates successful transplants with visible growth for as many generations as desired. A known strain of *B. influenzæ* was used as a control.

TABLE III.

Growth Requirements of No. C11 and B. influenzæ.

Strain No.	Meat infusion broth and ascitic fluid.	Meat infusion broth and unautoclaved yeast extract.	Meat infusion broth and ascitic fluid and unautoclaved yeast extract.	Peptone water and hematin, and unautoclaved yeast extract.	Peptone water and hematin, and yeast extract and ascitic fluid.	Hemo-peptone water.	Autoclaved hemo-peptone water.	Blood agar.
C 11	−	−	−	−	−	+	−	+
B. influenzæ.	−	−	−	+	+	+	−	+

− indicates that no viable organisms were demonstrated in the third transplant; + indicates that successful transplants were made for as many generations as desired. A known strain of *B. influenzæ* was used as a control.

the autoclave-labile factor to peptone water. Nos. C2, C4, C5, C6, C7, and C8 are similar to the influenza-like bacilli isolated from man (1) in that they reduce nitrates to nitrites, may or may not ferment certain sugars, are non-motile, effect no definite change on blood-milk, and require the addition of only the heat-labile factor as an accessory food substance. Neither of the organisms from man formed indole (1). Nos. C2, C4, and C7 do form indole. This, however, is no valid reason for not classifying these three strains with the parainfluenza bacilli (1), as this group also must be a large one comprised of Gram-negative bacilli with different cultural reactions but having in common the characteristic of requiring the addition of only the autoclave-labile substance as an accessory growth factor. From the results shown in Table II one might conclude that *Bacillus influenzæ* and No. C11 are alike. No. C11, however, ferments lactose. Since we had never seen a true influenza bacillus ferment lactose, an attempt was made to determine if No. C11 and *Bacillus influenzæ* differ in their growth requirements. From Table III it is evident that they do differ and that No. C11 is more exacting in its food requirements than the usual influenza bacillus. An organism similar to No. C11, so far as we know, has never been described. It is not like the other parainfluenza bacilli, yet rather than multiply names we shall include it with them provisionally.

DISCUSSION.

Wolff (11), working in Pfeiffer's clinic, isolated from a rat a Gram-negative bacillus which could not be differentiated from an influenza bacillus for 3 months. After this time, however, the bacillus acquired the property of growing on ordinary media. Friedberger (12), working in the same clinic, described a hemophilic bacillus from the preputial secretions of a dog. This bacillus has been studied by one of us (Rivers (9)) and found to be different from true influenza bacilli. M'Gowan (13), Torrey and Rahe (14), and Smith (15) have described *Bacillus bronchisepticus*. This organism should not be confused with the hemophilic group, still it seems that Mallory, Hornor, and Henderson (16), according to Smith (15), probably did confuse it with *Bacillus pertussis*. Organisms of the Pasteurella or hemorrhagic septicemia group form colonies similar to and look like influenza bacilli but should cause no trouble as they grow well on ordinary media. Park

and Williams (17) describe a method for differentiating the so called intermediate group of Gram-negative bacilli from *Bacillus influenzæ* and *Bacillus pertussis*. These intermediate bacilli grow on plain agar slants. The influenza-like bacilli from man reported in another paper (1) and the seven strains from cats described in this communication do not belong to any of the groups mentioned above. They will not grow on ordinary media unless some accessory growth substance is added and all of them so far studied, with the exception of No. C11, require the addition of only the autoclave-labile factor.

Recently Olsen (18) reported that he isolated from dogs sick of distemper bacilli which were identical with influenza bacilli culturally

TABLE IV.

Classification of Hemophilic Bacilli by Their Growth Requirements.

Bacterium.	Autoclave-labile factor.	Autoclave-stable factor.
B. pertussis (not recently isolated).	−	−
" *hæmoglobinophilus canis.*	−	+
" *influenzæ.*	+	+
" *parainfluenzæ.*	+	−

+ indicates the need of that particular food accessory factor; − indicates that the particular substance is not required. No. C11 does not belong to any of the groups and is more exacting in its food requirements than *B. influenzæ*. For convenience, however, it may be placed with the parainfluenza group.

and serologically. Since he did not go into details in regard to the methods of determining the growth requirements of the bacilli, and since there is a marked cultural and serological heterogeneity even among the true influenza bacilli, it is doubtful whether he was dealing with true influenza bacilli. We are inclined to think he might have been working with influenza-like bacilli similar to those we isolated from cats.

Since two accessory growth factors are necessary for true influenza bacilli, and since closely allied bacilli require neither or only one of these substances, Table IV shows how the so called hemophilic bacilli thus far studied might be classified according to their growth requirements.

The finding in animals of these Gram-negative bacilli which might be confused with influenza and pertussis bacilli on superficial examination is of importance to those who intend studying respiratory epizootics. The fact that such bacilli do exist should be fully recognized also by the workers who attempt to reproduce diseases in animals with *Bacillus influenzæ* and *Bacillus pertussis*. If the growth requirements and other characteristics are analyzed carefully no confusion should result.

CONCLUSIONS.

Six strains of Gram-negative non-motile bacilli from cats have been described. They do not grow on ordinary media. They do grow, however, on a medium to which an autoclave-labile substance alone has been added as an accessory growth factor. These bacilli are similar to *Bacillus parainfluenzæ* isolated from man. One strain of a Gram-negative non-motile bacillus more exacting than *Bacillus influenzæ* in its food requirements was found and for convenience has been placed for the present in the parainfluenza group.

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STUDIES ON ENDOTHELIAL REACTIONS.

VII. Changes in the Distribution of Colloidal Carbon Noted in the Lungs of Rabbits Following Splenectomy.

By NATHAN CHANDLER FOOT, M.D.

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Plates 9 and 10.

(Received for publication, August 22, 1922.)

INTRODUCTION.

As described in the preceding article of this series (Foot, 1922), it appears that the spleen, liver, and bone marrow play the most important part in the production of the macrophages found free in the meningeal vessels of rabbits affected with experimental tuberculous meningoencephalitis. The lungs also show slight proliferative changes of the endothelium, but this might be due to the stimulating effect of the injected ink, or the general tuberculous infection. One means of more sharply localizing the source of these macrophages would be to extirpate the organ, or organs suspected of producing them and to observe what happened as a result. Of the organs named, the spleen alone could be extirpated, and this was done, in the expectation that the liver and bone marrow, or the lymphatic organs, would compensate for the loss, or else that the supply of circulating macrophages would be reduced materially.

However, when the organs just mentioned were examined after splenectomizing these tuberculous rabbits, it was found that the lungs showed profound and uniform changes, while the liver, bone marrow, and lymphoid tissue remained in a condition quite similar to that observed in control animals. It is generally accepted that by far the greater part of the colloidal ink injected into the veins of experimental animals is to be found distributed in the liver, spleen, bone marrow, and lungs, in the order named. Drinker (1921) and

his associates found this also to be true in the case of injections of manganese dioxide, with the exception of one animal out of a series of mammals, birds, and amphibians, that animal being the cat. In the last instance, this material was, at first, largely absorbed by the lungs, the other organs taking up a far smaller percentage. Later the manganese became redistributed and the amount found in the lung fell below that determined to be present in the liver.

After removing the spleen of the rabbit it is readily seen at necropsy, on gross examination, that the distribution of the intravenously injected ink has been altered. The lungs are far blacker than those of control animals, tuberculous or otherwise, the liver somewhat paler, while the bone marrow appears to contain the usual amount. This is readily confirmed by the microscope; the ink is found to be taken up by innumerable macrophages in the lung, both *in situ* in the endothelium of the capillaries and larger vessels and lying grouped in the lumina of the capillaries, whose walls are thickened and show ample evidence of proliferation. The liver, on the other hand, while it shows a liberal distribution of ink in cells in the sinusoids, is no more heavily charged than in the controls. As for the bone marrow, the endothelium contains no more ink than does that of the controls.

EXPERIMENTAL.

This being the case, it would be well worth while to follow up such a lead in a series of animals under more normal conditions, in which there is no tuberculous infection and to compare them with the series already briefly described. The rabbits used in this experiment are divided into four groups: a control group (A) of three animals, a second group (B) composed of the four animals from the experiment on cerebral tuberculosis, a third group (C) of three splenectomized, non-infected rabbits, and a fourth group (D) comprising two splenectomized rabbits into which manganese was injected intravenously, instead of ink.

Group A.—Three rabbits were used for controls, one of them receiving 5 cc. of 50 per cent Higgins' waterproof ink in distilled water intravenously, followed by similar doses 3 and 5 days later, making a total of 15 cc. of the ink mixture. It was killed 2 hours after the last dose. The dosage of the other two rabbits was regulated by that of the first pair of animals in Group B; they received 5 cc.

into an ear vein, followed by three similar doses at intervals of 4, 7, and 11 days, and were killed by chloroforming immediately after the last dose had been administered. They showed no ill effects from the injection.

At necropsy the first and third rabbits showed the usual changes following ink administration: lungs gray, or iron-gray, liver black, spleen black, bone marrow black, and kidneys grossly unchanged. The lungs of the second rabbit, however, were black, the liver, spleen, and bone marrow black, and the kidneys unchanged.

Group B.—Four rabbits were splenectomized under complete anesthesia and bovine tubercle bacilli injected, through a trephine opening, into the right cerebral hemisphere.[1] They then received intravenous injections of 5 cc. of 50 per cent Higgins' ink in distilled water a few days after the operation. The dose was repeated without untoward effects on the 4th and 7th days, but upon administration to two of the rabbits on the 11th day after the first dose, both died within a few minutes with symptoms suggesting cerebral embolism. The other two rabbits received a fifth dose 2 weeks later, which caused no trouble, but upon injecting a similar amount of ink mixture into one of them a week later, it died precisely as had the other two. The fourth rabbit was then allowed to go uninjected and was killed 8 weeks after the administration of the initial dose, the object being to allow time for the redistribution of the injected material and the possible removal of absorbed ink from the lungs to other organs whither it might be traced.

The gross findings at necropsy, in this group, have already been described in the introduction. Apparently we have an interesting effect of splenectomy on these rabbits; namely, inability to handle the injected ink after a certain amount has been given, with consequent death from cerebral embolism. Ink emboli were found in the cortical precapillaries of all three animals dying after injection, none being found in the fourth, which was killed by chloroform and the intra-arterial injection of Zenker's fluid. Such deaths from ink embolism are very unusual; it has happened but two or three times in the course of some hundreds of injections and then usually only when the ink was administered in far larger quantities. It is obvious that the amounts of ink given the splenectomized animals were moderate and that the injections were separated by ample intervals of time. In view of the fact that the experiment was repeated in exactly the same way, with the omission of the injection of tubercle bacilli and cerebral injury, in Group C, without a single death, it would appear that cerebral tuberculosis is the determining factor for this phenomenon, although why this should be so remains unexplained.

Group C.—Three rabbits were splenectomized and the ink mixture was given as just described in the preceding group. One rabbit was killed 1 hour after the first injection, to determine the immediate distribution of the ink; the other two were injected in precisely the same manner as were those of Group B which died after the fourth injection. In the case of these two rabbits no ill effects were

[1] See Paper VI (Foot, 1922).

noted after four injections of 5 cc. each of the ink mixture, other than slight dyspnea. 3 days later each was given 10 cc. of the mixture, intravenously, the dose being repeated 2 days later without any bad result, making a total of 40 cc. instead of 20 cc. of the mixture of ink and distilled water. The rabbits were killed with chloroform a few hours later, and their tissues were fixed immediately in Zenker's fluid. The same bottle of ink was used for these larger injections that was employed for the four rabbits in Group B.

All three animals showed a blackening of the lungs and liver, the 1 hour rabbit's bone marrow was brownish red, that of the other two black. The kidneys of the 1 hour rabbit showed no gross change, those of the others were grayish in the cortical zone.

Group D.—In order to determine the distribution of particulate matter after the lapse of an hour, in such splenectomized animals, and to compare it with that in normal rabbits, the following experiment was performed.[2] Two splenectomized rabbits were anesthetized and injected with salts of manganese suspended in Ringer's solution. One of them received 20 cc. of a suspension of manganese dioxide, containing approximately 2.1 mg. of Mn, into an ear vein. The other was given 35 cc. of a similar suspension of Rhodonite (manganese metasilicate), containing about 2.1 mg. of Mn. Analysis of the liver and lung, after 1 hour, showed 0.76 per cent Mn in the lung, in the case of the dioxide and 1.02 per cent in that of the Rhodonite. The livers showed 98 and 61.8 per cent respectively. These figures do not deviate from the normal sufficiently to show any difference attributable to the splenectomy. Microscopic examination shows a very different picture from that obtained after the administration of Higgins' ink. There is no visible reaction to the presence of the manganese particles, on the part of the endothelium, showing that they are much less stimulating than the ink globules. It is therefore doubtful whether the cases are parallel. For a description of the technique used in this group the reader is referred to the article by Drinker and Shaw (1921).

The Altered Histology in Splenectomized Animals.

Lungs.—As compared with control specimens (Fig. 1), the lungs of the splenectomized rabbits show certain definite changes. On the whole, there is a resemblance to the lung of chronic passive congestion; the alveolar walls are thickened and the capillaries tortuous and engorged with innumerable mononuclear cells containing ink in quantities sufficient to mask completely everything but the nucleus (Figs. 2 and 3). The thickening of the walls is due to a swelling of the endothelium of the capillaries and to a lesser degree to an accum-

[2] Mr. Louis A. Shaw, of the Department of Industrial Medicine of this School, kindly performed this experiment.

ulation of primitive, mesenchymoid cells in the interstitial tissue. Furthermore, the endothelium is liberally besprinkled with particles of ink. The nuclei of the capillary endothelium are swollen and increased in number, the cells crowded together with their nuclei forming chains. Mitotic figures are present, though rather difficult to find, situated in the sessile endothelial cells of vessels. Amitosis is suggested by the presence of large, lobulated nuclei, resembling those of tumor giant cells (Fig. 4), such as occur so frequently in Hodgkins' lymph nodes. What their presence denotes is not clear; they are encountered occasionally in the lungs of control rabbits.

The ink-laden macrophages tend to clump together in masses in the large capillaries that are seen, in cross-section, projecting into the alveoli. They often occlude these vessels without, however, causing any inflammatory changes in their walls (Fig. 5). There is no fibrin, nor are there masses of blood cells or platelets; apparently these plugs are quite inert. They have persisted in this situation 8 weeks after the first injection, 6 weeks after the last, in one rabbit of Group B. Some of these cells migrate to the peribronchial lymphatics (Fig. 6), but the majority remain in the capillaries. It is highly probable that they are no longer composed of discrete cells, but of cells fused to form syncytial masses. This process is more clearly seen in the liver; in the lungs it is difficult to make out the cell boundaries on account of the large amount of ink in the cytoplasm of these clumps. The cells containing ink vary from small forms to very large, mulberry-like types. All stages between the two are to be found; some cells contain but little ink, others a moderate degree, while others are engorged with it. The smaller the amount of ink, the smaller the cell is, as a rule; the larger the number of globules of ink, the larger the cell. It is not possible to divide these cells into two clear-cut types by means of the methods employed here; that they are composed of two such classes, monocytes and macrophages (Simpson, 1922), seems unlikely. It appears that they represent the same cell in various phases of development or repletion. The larger vessels show many free, ink-laden macrophages and few free from ink, if the lung be examined shortly after injection; in lungs observed 6 weeks after the last injection many of the larger veins are thronged with compact mononuclear leucocytes resembling Simpson's monocytes and free from ink.

Besides these, there are a good many ink-bearing cells still free in the larger vessels (Fig. 7). These contain varying amounts of ink. The only other cells in the lung found to contain ink at this time are those blocking the larger capillaries and the sessile endothelium of the pulmonary capillaries in general. One may infer from this that the ink bearers are thrown off gradually into the larger vessels by the endothelium.

The alveolar spaces of the lungs in these rabbits are, for the greater part, narrowed and compressed by the thickening of their walls and by the encroachment of capillaries engorged with ink-laden macrophages. Here and there compensatory emphysema makes up for this diminution in the air capacity. The macrophages, when ink-laden, do not tend to enter the alveolar spaces to any marked extent; they either remain in the capillary plugs already described, or migrate to the peribronchial lymphatic tissue (Fig. 8), where they form black masses in the peripheral sinuses of the nodes, or they appear to be thrown off into the blood stream to lodge elsewhere, a phenomenon which will be discussed later.

The microscopic appearance of the lungs of the rabbits in Group C does not differ from that in Group B, except that those receiving larger amounts of ink (40 cc.) show greater numbers of ink-bearing cells and larger clumps of these cells in the capillaries. 1 hour after one injection of 5 cc. the lungs already contain an abnormal amount of phagocytosed ink, as well as emboli, or plugs, of uningested carbon (Fig. 9).

Liver.—The livers of these animals do not show a very marked deviation from the normal standard established in the controls (Figs. 10 and 11). Two facts, however, stand out rather clearly. In the splenectomized animals there are numerous mitotic figures in the cells of the parenchyma, one of which is shown in Fig. 11, and the macrophages show a marked tendency to be rounded in form with the ink distributed peripherally in their cytoplasm. Little ink is seen in the endothelium of the sinusoids, as compared with that of the controls. The ring-forms, with peripherally distributed pigment, have been described by Evans, Bowman, and Winternitz (1914) and by McJunkin (1919), in non-splenectomized animals; they seem to be more numerous in the splenectomized rabbits. After 6 weeks the ink-bearing macrophages show a tendency to migrate to the lym-

phatics of Glisson's capsule and there to form syncytia. Some ink penetrates the liver cells, as has been noted by various investigators and by the writer (Foot, 1921).

Bone Marrow.—Here, too, if there be any deviation from the control standards, it is in the direction of a diminution in the amount of ink found in the endothelium of the marrow. The cells containing it tend to form the same rounded clumps mentioned in the case of the lung and liver, losing their reticular arrangement. In controls the reticular form is retained, the cells remaining elongated, polygonal, and anastomosing. How this variation is to be interpreted is not clear; it is certain, however, that the marrow does not compensate by increased phagocytic activity for the loss of the spleen.

Kidneys.—In the kidneys the macrophages gather at first in the glomerular tufts, in rounded or elongated masses; in later stages the glomeruli show less and less ink, while the endothelium of the intertubular capillaries shows increasing amounts. There is somewhat more ink in these kidneys than in those of the controls, but the difference is not marked.

Brain.—Very little ink is absorbed in this organ and, as time goes on, it is difficult to find it anywhere except widely and sparingly distributed in the meningeal capillary endothelium. In those rabbits dying from the injection of ink, the precapillaries of the cortex are found plugged with ink emboli, which are unassociated with cellular structures; apparently a purely mechanical agglomeration of carbon particles has taken place. These plugs are very numerous and seem to account for the death of the animals more clearly than do the cellular plugs in the pulmonary capillaries; the former are found only in the animals that died after injection, not in any of the others examined, while the pulmonary plugs are found in all of the rabbits injected with ink.

Abnormal Ink Distribution in a Control Animal.—The second control rabbit in Group A shows a peculiar condition. The lungs are grossly black; microscopically they are found to be thickened conspicuously, the endothelium is swollen and ink-laden, and the interstitial tissue is much increased. The liver contains less ink than normally and the spleen very much less. The kidneys also show more ink than usual. It is apparent that the animal has had some ante-

cedent pulmonary infection, possibly snuffles, resulting in a great increase in interstitial tissue at the expense of the alveolar spaces. The cells of this tissue are primitive in type, swollen, and have taken up abnormal amounts of ink; at the same time the spleen has absorbed much less than normally. This can be interpreted in two ways —either the spleen, in failing to withdraw a normal amount of ink from the circulation has thrown it into the lungs, precisely as in the case of the splenectomized animals, or the pulmonary endothelium has become more actively phagocytic and withdrawn the ink before the spleen could do so. There is more evidence in support of the latter theory; the spleen seems to be normal, from the histological standpoint, and the lungs are definitely abnormal and appear to have been the site of an inflammatory process.

DISCUSSION.

The literature on experimental splenectomy will be found ably reviewed by Pearce (1918), who traces the history of the operation back to the ancient Greeks and brings it up to modern times. There is little in this literature bearing upon the absorption of foreign material from the blood after the spleen has been removed. Most of the work done has been focused upon the spleen as an activating organ for biliary, gastric, and pancreatic secretion, or for the regulation of peristalsis, or the appetite and rate of growth.[3] This is because Schulze, in 1828, believed that he had produced alterations in the secretion of bile by splenectomy and because the spleen becomes swollen during digestion. Pearce quotes from the various investigators at some length. His own experiments are concerned with the effect of splenectomy upon the biliary secretion and hemolysis, in sensitized animals. He finds that the lymph nodes, hemolymph nodes, and bone marrow assume the function of phagocytosing disintegrated red cells, after splenectomy, and believes that they also aid this organ, normally, "in times of stress." He also mentions several investigators who have found such compensatory hypertrophy of the lymphatic apparatus. Nowhere is the lung mentioned as a compensatory phagocytic organ. There is nothing in the present experiment to show

[3] See also Mollow (1921), Prym (1911), and Smith and Ascham (1921–22).

that Pearce's observations on the phagocytosis of erythrocytic debris have any bearing upon the fate of colloidal ink, a very different substance; the lymph nodes do not compensate within 8 weeks for the loss of the spleen, nor does the bone marrow.

Drinker and Shaw (1921), however, stress the primary importance of the liver as a phagocytic organ; even in the cat, after a preliminary 3 hour phase of phagocytic activity in the lung, the liver ultimately appears to secrete the injected manganese into the intestinal tract, after the material has been redistributed from the lung. They believe that there is an essential difference in the phagocytic properties of the capillary beds of the lung and liver and find that the bone marrow plays but an unimportant part in the sterilization of the blood stream, a fact borne out by their subsequent investigations with Lund (1921). There is some confirmation of their hypothesis as to the importance of the liver as the penultimate depository for particulate matter (the alimentary tract being the ultimate one) in the findings in the present experiment; redistribution from lung to liver is strongly indicated, the presence of ink globules in the liver parenchyma of animals in which the ink was forced, or in which some time elapsed after its administration, is significant. The presence of mitotic figures in the liver epithelium also indicates activity on the part of the parenchyma of this organ. That the bone marrow plays a minor rôle is also confirmed. The experiment with Group D shows that manganese does not behave like the ink within 1 hour's time, a fact that warns one not to attempt to draw too close a parallel between the effects of the two substances.

Two articles that have appeared recently are also of interest. Simpson (1922) reports that the mere injection of Higgins' ink will call forth "showers" of ink-laden macrophages in the circulation, more often and more copiously in the venous and pulmonary than in the systemic. She concludes that they are poured out by the spleen and liver, or that they are backed up from the clogged pulmonary capillaries. She accepts Kiyono's (1914, *a*, *b*) evidence that they are produced by the endothelium or the reticuloendothelium of the hematopoietic organs, the "specific endothelia" of Evans (1915). It is hard to account for the origin of the macrophages found in the lungs in the present experiment if it is not the pulmonary endothelium

itself; the spleen has gone, the liver shows little or no activity in the sinusoidal endothelium, and the reticulum of the bone marrow is not proliferating. Such proliferation is marked in the pulmonary endothelium, as evidenced by swelling, increase in the number of nuclei, and the presence of mitotic figures.

The other article, by Westhues (1922), refers to the lung as a filter bed for the macrophages produced elsewhere in the body. He belittles the phagocytic capacity of the pulmonary endothelium, basing his assumption upon experiments carried out with mechanically suspended India ink. He either overlooks, or misunderstands the importance of adding an emulsifying agent (such as gelatin or acacia) to the ink, a fact repeatedly stressed by McJunkin (1919), the originator of this technique, and by myself (Foot, 1920, 1921). He also appears to have missed Permar's (1920–21, *a*, *b*, *c*) excellent articles on pulmonary phagocytes. A glance at Fig. 2 will show that the endothelium of the lung is actively phagocytic for such a colloidal suspension.

SUMMARY.

The changes in the distribution of intravenously administered colloidal ink in splenectomized rabbits may be interpreted somewhat as follows: The removal of the spleen throws an increased amount of ink into the other hematopoietic organs and the lung and liver. While we should expect the liver or the bone marrow to compensate for the loss of the spleen and to take up this ink and remove it from the circulation, that is not the case. The lungs appear to play the chief part in the process, slowly passing on the removed material, contained in macrophages, to the liver, or retaining these ink-laden cells in their tissues and capillaries. By an increase in the capillary endothelium and by a process of engorgement of the capillaries with cells presumably derived therefrom, the lungs remove by far the greater part of the foreign material that has been introduced into the circulation.

At the same time there is an increase in the number of ink-bearing macrophages in the lymphatics and capillaries of the lung, indicating that these cells are entering the circulation and the lymph stream (Fig. 8). The only organ where they lodge in any quantities, outside

of the peribronchial lymph nodes, is the liver, the sinusoids of which contain an increasing number of macrophages as time goes on. It is possible that these cells are destroyed in the sinusoids and the carbon transferred to the liver epithelium; there is evidence to support this assumption. After the lungs, the liver comes next in degree of intensity of pigmentation; the bone marrow contains far less than either of these organs.

CONCLUSIONS.

1. After splenectomy in rabbits colloidal carbon introduced into the circulation is removed primarily by the lung, which compensates for the loss of the spleen and contains vastly more carbon than that of a normal rabbit.

2. The liver, bone marrow, and peripheral lymph nodes show no marked alteration in their phagocytic activity, as compared with those of controls. They do not compensate for the loss of the spleen.

3. The cells phagocytosing colloidal carbon in the lung appear to be produced there, rather than in other organs, as proliferation of the endothelium occurs chiefly in the lung under these conditions.

4. These cells either remain in the pulmonary capillaries and lymphatics, or are thrown into the circulation.

5. In the latter case there is an apparent increase in the number of macrophages in the lumina of the liver sinusoids, but nowhere else, indicating a transference of carbon from lung to liver within cells.

6. Hence it is probable that these cells are destroyed in the liver and their content of carbon taken up by the parenchyma, since the latter frequently contains carbon particles and shows mitotic activity.

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EXPLANATION OF PLATES.

Histological material fixed in Zenker's fluid, stained either with Mallory's eosin-methylene blue or with Delafield's hematoxylin and eosin.

PLATE 9.

FIG. 1. Control lung, after four injections of 5 cc. of Higgins' waterproof ink and distilled water, 50 per cent, intravenously. No splenectomy. Ink moderate in amount, tending to be scattered rather diffusely through the endothelium and lying in masses in the capillary lumina. Hematoxylin-eosin. × 275.

FIG. 2. Lung from a rabbit in Group B after splenectomy. The same amount and concentration of ink were injected intravenously as in the preceding animal. There is a great increase in the amount of ink present; note the ring-like distribution. Eosin-methylene blue. × 275.

FIG. 3. Lung from another rabbit in Group B; the picture is quite similar to that in Fig. 2. Note the macrophages in the vessel at the left upper corner. Eosin-methylene blue. × 275.

FIG. 4. Large lobulated nuclear mass near the center described in the text, 6 weeks after the last injection of ink. Dosage the same as the preceding, but extended over a longer period of time, with greater intervals. Eosin-methylene blue. × 350.

FIG. 5. The same rabbit as in Fig. 4. Note the size of the cell masses containing ink and compare with those in Figs. 2 and 3. The endothelium and lumina of the smaller capillaries are almost free from ink. Eosin-methylene blue. × 275.

FIG. 6. The same rabbit. Lymphatics clogged with ink-laden macrophages alongside a bronchiole. Eosin-methylene blue. × 275.

PLATE 10.

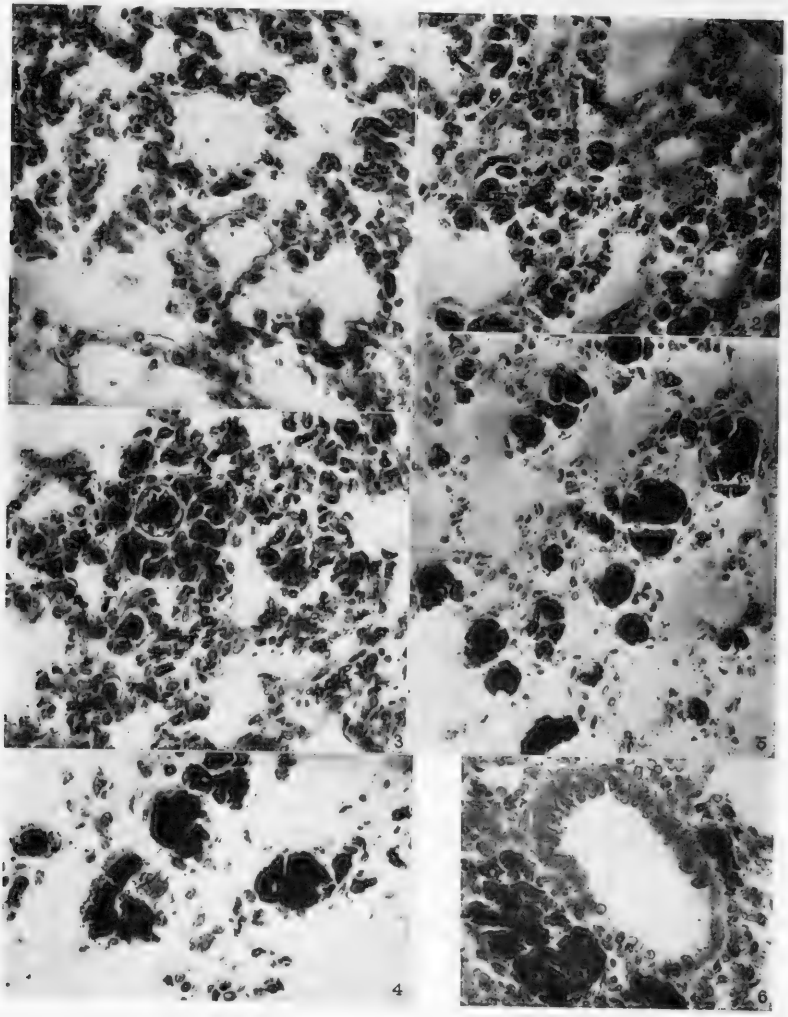
FIG. 7. A pulmonary vessel containing many ink-laden macrophages. Hematoxylin-eosin. × 275.

FIG. 8. The same rabbit as in Figs. 4, 5, and 6. Lymph vessels at the periphery of a peribronchial lymph node. They are choked with macrophages. Eosin-methylene blue. × 275.

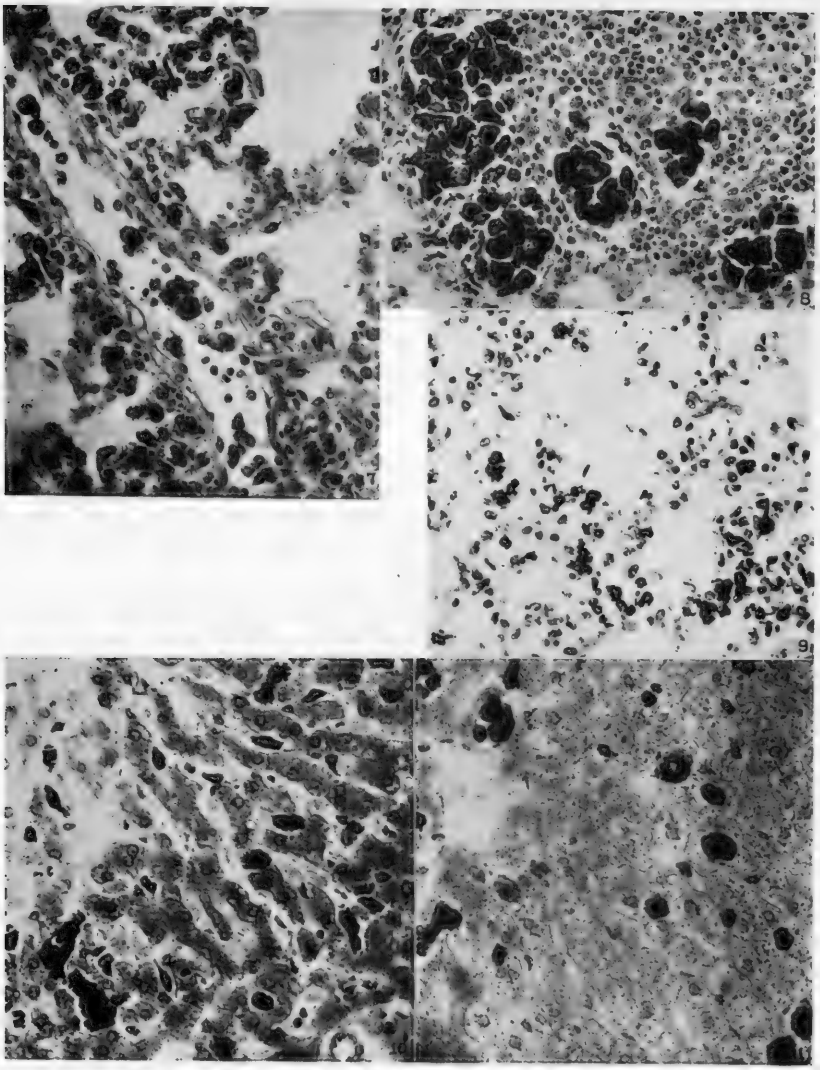
FIG. 9. The lung 1 hour after injection of 5 cc. of Higgins' ink suspension into the ear vein of a rabbit in Group C. More ink is present than in controls not represented in the illustrations. Eosin-methylene blue. × 275.

FIG. 10. Control liver from an animal in Group A, after 15 cc. of ink had been injected intravenously. The cells containing ink are elongated. Eosin-methylene blue. × 275.

FIG. 11. Liver from a splenectomized rabbit (the same animal as in Fig. 2). Here the ink is in rounded masses in cells lying free in the sinusoids. Note the mitotic figure at the upper center and the fact that 20 cc. of ink were injected into this animal as against 15 in the preceding one. Eosin-methylene blue. × 275.



(Foot: Endothelial reactions. VII.)



(Foot: Endothelial reactions. VII.)

THE SOURCE OF AGGLUTININS IN THE MILK OF COWS.

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(Received for publication, August 2, 1922.)

For several years the milk of cows has been under investigation in this Department with special relation to the udder as a locus of multiplication of *Bacillus abortus* and the bearing of this condition on infection of the placenta during gestation. The presence of this organism in the udder has been detected by inoculating guinea pigs with the concentrated sediment of milk and by plating portions of the same. At the same time the samples of milk have been subjected to the agglutination test. In the majority of cases the agglutinins in the milk presented that fraction of the blood agglutinins which has been usually ascribed to a filtration from the blood. Certain cases, however, could not be ranged in this group. The ratio of agglutinins in the milk to that of the blood serum was far higher than that due to such assumed filtration. Thus in the first class of cases this ratio fluctuated between 1/16 and 1/64. In the second it ranged from 1/4 to 1/1. In samples of colostrum taken soon after parturition it ranged from 1/1 to 2/1. Here the high concentration is in part due to a slow accumulation in the inactive udder for within a few days after the active secretion of milk has begun the agglutinins may fall to the 1/16 or 1/32 ratio. In a few cases they have remained high. Two instances taken from experiments made for another purpose illustrate the differences which may be encountered (Table I). Both cows were vaccinated by a subcutaneous injection of living bacilli. Although both reacted to the inoculation with an accumulation of agglutinins up to the same high level in the blood serum, the milk showed in one case a low output, in the other a high output of

TABLE I.

Cows A843 and 585. Vaccinated with Living Bacilli May 7, 1921.

Date of blood sample.	Blood serum titer.	Date of milk sample.	Milk titer.
	Cow A843.		
1921		*1921*	
		Apr. 21	1:20
May 1	1:40		
" 10	1:40	May 10	1:20—
" 17	1:1,280+	" 17	1:20
		" 24	1:20
" 31	1:2,560	" 31	1:20
		June 7	1:20
June 22	1:1,280+	" 21	1:20
		July 11	1:20—
		Sept. 26	1:10
Nov. 8	1:1,280	Nov. 16	1:20—
1922		*1922*	
Jan. 25	1:320+	Jan. 25	1:40—
	Cow 585.		
1921		*1921*	
		Apr. 21	1:20
May 1	1:40		
" 10	1:80—	May 10	1:20—
" 17	1:1,280	" 17	1:40+
		" 27	1:40
" 31	1:2,560	" 31	1:40
		June 7	1:20
June 22	1:1,280		
		July 11	1:40
		Sept. 26	1:320+
Nov. 8	1:1,280+	Nov. 18	1:640
		Dec. 12	1:640
1922		*1922*	
		Jan. 6	1:1,280
Jan. 25	1:1,280	" 25	1:640

agglutinins. The stage of lactation was about the same in both animals.

The second animal (No. 585), a cow weighing about 1,000 lbs. and secreting about 28 lbs. of milk on January 6, was discharging daily

through the udder fully one-third of the agglutinin content of the blood. The only demonstrated difference between the two cases was the presence of living *Bacillus abortus* in the milk of No. 585. A large series of cases tested on guinea pigs has shown that a low agglutinin titer of the milk is not associated with *Bacillus abortus* in the udder, whereas from many animals with a high agglutinin content, *Bacillus abortus* was isolated from the milk through guinea pigs. In the few negative cases probably repeated tests would have revealed the bacilli.

Two alternatives presented themselves to be examined experimentally. Either there is a local production of agglutinins in the udder tissue or else the udder tissue is made completely permeable to the blood agglutinins in the presence of *Bacillus abortus*.

EXPERIMENTAL DATA.

To interpret the effect of injecting bacteria, dead or living, into the udder ducts the relation existing among the different parts of the udder through the ducts should be kept in mind.

The udder of the cow is divided into two anatomically independent lateral halves, bound closely together by connective tissue. They arise from two separate anlages in the embryo. Each half is divided into two quarters and each quarter has its group of ducts and its own teat canal. The parenchyma of each pair consisting of fore and hind quarter shows no dividing line but appears to form a single secreting mass. The quarters are, however, relatively independent, for infectious processes are, as a rule, restricted to one quarter at the start. In what follows concerning the local production of agglutinins it is of importance to know whether fore and hind quarters are in communication, even though restricted, with one another through their ducts. To throw light on this a pathological reaction was brought into play. When suspensions of *Bacillus abortus* are injected into a quarter there is a prompt reaction in that quarter, manifested locally by swelling of the entire quarter and a copious emigration of polynuclear leucocytes into the milk. This is at its maximum within 24 hours.

A cow about to be slaughtered, No. 601 of the experiments to be detailed, received a suspension of *Bacillus abortus* into the left fore quarter at 4 p.m. The cow was killed next day at 3 p.m. and the udder removed and portions of the four quarters fixed in Zenker fluid.

The microscopic examination of the injected quarter showed a very extensive filling up of the secreting alveoli with polynuclear leucocytes. The same kind of cells permeated the epithelium and the interstitial tissue where this was appreciable in extent. The other quarters, including the left hind quarter, showed some slight focal infiltration which is probably to be accounted for by the presence of staphylococci and streptococci found in earlier studies of this case. It is highly probable, therefore, that if any communication exists between the ducts of the fore and hind quarters it is very restricted.

The suspension was injected into an udder duct as follows: A milking tube was chosen long enough to pass through the teat canal into the cistern. To this a glass syringe holding 25 cc. was attached by means of a rubber tube. This outfit was sterilized. The udder was washed and left partly moist. After the introduction of the milking tube, some milk was allowed to drain out and then the culture injected. The teat canal was compressed for 5 minutes to prevent escape of the fluid.

Because of the character of the milk it cannot be used in its native condition for agglutination tests since it is impossible to distinguish in tubes in the usual way the clumping in low dilutions. To clarify the milk rennet has been found satisfactory. Unless the cream is taken off first, the whey which separates out after clotting with rennet is clouded. Therefore, the milk is centrifuged and the cream removed. The tube of milk is then put into a water bath at about 35°C. A rennet tablet is dissolved in about 10 cc. of sterile distilled water and 8 to 10 drops added to the slightly warmed milk, the whole shaken and left standing for 10 or 15 minutes to coagulate. It is then put in a water bath at about 45–50°C., which causes the coagulum to shrink and a water-clear whey to separate out. This whey is used for the agglutination test as is blood serum and the dilutions made with normal salt solution.

I. The Injection of Living Bacilli into the Udder.

The first experiment attempted was to inject a suspension of living bacilli directly into the ducts of one quarter of the udder to verify inferences based on spontaneous cases.

Cow 523.—Calved July 9, 1919, and Sept. 2, 1920. In October she reacted to the tuberculin test.

Agglutinin tests on record before the experiment was started were: Oct. 22, for the blood somewhat above 1:40, for the milk on Oct. 22 and Nov. 8 about 1:10.

The flora of the udder was looked into before inoculation. On a blood agar plate colonies of hemolytic streptococci appeared which, treated with an immune serum towards bovine hemolytic streptococci, were agglutinated completely at 1:1,280. There were about 17,600 in a cubic centimeter of milk. Only a small per cent of colonies of other bacteria were on the plate.

On Nov. 8, 1920, after having been milked out the right fore quarter was inoculated by an injection into the milk duct of 18 cc. of a suspension prepared as follows: *B. abortus* isolated Oct. 28 from the meconium of a fetus (No. 460) was cultivated on a layer of agar in a Blake bottle sealed with sealing wax for 3 days at 37°C. The growth was washed off, suspended in normal saline, and diluted to a density of 2.4 as measured by the Gates instrument. This dilution corresponded to approximately 3,300 million bacteria per cc.

Considerable attention was given both during milking and in the intervals to prevent contact infection of the other quarters. After several weeks this care was discontinued because of the greater opportunities for infection by way of the blood.

Next day, the cow was uneasy and refused her food. The temperature at 7 a.m. was 41°C., at 1 p.m. 41.6°C. The *R. F.*[1] was swollen to twice its normal size. It was warm and sensitive to manipulation. The fore milk was thick and flaky. The total secretion of milk fell from 9 to 3 quarts. The agglutinin titer of the milk had not risen.

Plate cultures of the milk showed a decided diminution of streptococci in the injected quarter. Compared with the colonies obtained before inoculation they had been reduced 400 times. It was at first supposed that the injection of *B. abortus* had cleared the way for a streptococcus mastitis, but the platings showed, on the contrary, that the mastitis was due to *B. abortus*.

On Nov. 10, the cow was not eating the normal amount of feed. The temperature at 7 a.m. was 40°C., and at 1 p.m. 40.6°C. The injected quarter was still swollen and firmer than yesterday. The swelling was extending forward. The fore milk was thick and yellow. The total yield was 4 quarts.

On Nov. 11, the general condition of the cow and udder was the same but the temperature was lower, 39.2°C. at 7 a.m. and 38.6°C. at 5 p.m. Samples of fore, middle, and last milk from the four quarters were tested and since the difference in agglutinins was inappreciable only the middle milk was tested. The *R. F.* was now 1:80, the L. F. 1:20, the others below 1:20. The blood titer was between 1:40 and 1:80.

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[1] R. F., R. H., L. F., and L. H. stand for right fore and right hind quarter and left fore and left hind quarter respectively. The italics indicate the injected quarter.

On this day a distinct increase in the number of hemolytic streptococci in the injected quarter was evident. There were, per cc., 1,105, 920, and 250 colonies in the fore, middle, and last milk respectively.

On Nov. 12, the cow was not eating normally. The udder was swollen, the milk not so thick, and the total more abundant.

On Nov. 14, there was slight improvement in all directions. The titer of the milk was as follows: *R. F.* 1:80, the rest 1:20. Colonies of hemolytic streptococci per cc. were distributed as follows: *R. F.* 350, L. F. 230, R. H. 1,730, L. H. 4,020.

The milk titer on the 10th and the 11th day showed an increase in agglutinins towards *B. abortus*:

	Nov. 18	Nov. 19
R. F.	1:320	1:640
R. H.	1:80	1:320
L. F.	1:160	1:320
L. H.	1:80	1:320

Guinea pigs inoculated with last milk of *R. F.* Nov. 19 subsequently proved negative to *B. abortus*.

On Nov. 21, the only abnormalities observed were the still swollen quarter and its reduced secretory activity. The agglutinin titer was as on the 19th. Unfortunately the agglutination test of the blood had not been taken since Nov. 11 when it was not quite 1:80. On Nov. 21 it was 1:1,280, having risen to this high level in the meantime.

On Nov. 29, the blood titer had risen but that of the milk had declined. The blood titer was 1:2,560, *R. F.* 1:160, the rest 1:80. The hemolytic streptococci were gradually gaining ground: *R. F.* 6,550, R. H. 12,150, L. F. 6,520, and L. H. 7,620 colonies per cc.

On Dec. 9, 31 days after the inoculation, the milk titers had again risen slightly: *R. F.* 1:320, the rest 1:160. On Dec. 16, the milk titers were as on Dec. 9. On Dec. 27, 49 days after inoculation, *R. F.* was still indurated and not secreting as much as before the inoculation. The milk titers shifted somewhat in that the injected quarter rose and the others fell: *R. F.* 1:640, the rest 1:80. The blood titer was high, 1:2,560.

On Jan. 14, 1921, the 67th day of the experiment, the udder conditions were as before. The milk titers shifted slightly: *R. F.* 1:640, R. H. 1:80, the others 1:40 or less. The blood titer was 1:2,560. The colonies of hemolytic streptococci were still increasing in numbers: *R. F.* 7,800, R. H. 9,600, L. F. 9,000, and L. H. 14,200 per cc.

On Jan. 26, the 79th day of the experiment, the milk titers were: *R. F.* 1:320, the rest 1:40. The colonies of hemolytic streptococci per cc. were as follows: *R. F.* 8,900, R. H. 4,600, L. F. 900, and L. H. 10,400. Two guinea pigs inoculated with milk from the injected quarter were found free from *B. abortus* later on.

Second Injection.—On Jan. 27, a similar injection of a living *B. abortus* sus-

pension was made into the left fore quarter of the udder. The cow reacted during the following 24 hours much as before both as regards the general condition and the udder itself.

On Jan. 28, the agglutinins in the injected quarter had risen promptly: *L. F.* 1:160, *R. F.* 1:640, R. H. 1:40, L. H. 1:40. At the same time the agglutinins in the quarter injected first rose again and maintained a high level throughout.

The hemolytic streptococci had shifted as a result of the injection.

	Before injection.	After injection.
L. F.	900	0
R. F.	8,900	11,800
R. H.	4,600	1,700
L. H.	10,400	15,700

They had practically disappeared from the injected quarter in which an acute mastitis developed.

On Jan. 29, 2 days after the injection, the cow still refused food and the injected quarter was twice its normal size, firm, painful, and warm to the touch. The fore milk was thick and yellow.

The agglutinin titer was higher: *L. F.* 1:320, *R. F.* 1:640, the others 1:160. The hemolytic streptococci were reappearing in the injected quarter and were present in the right fore quarter injected first in larger numbers than ever: *L.* F. 300, *R. F.* 30,100, R. H. 4,500, L. H. 20,100 colonies per cc.

On Jan. 31, 4 days after the injection, the titers were as follows: *L. F.* 1:640, *R. F.* 1:640, R. H. and L. H. a trifle below 1:80; the blood titer was 1:2,560.

On Feb. 6, the cow's appetite was improving. The injected left fore quarter was still large and indurated and the fore milk thick and yellow. The titers were slightly lower: *R. F.* and *L. F.* 1:320, R. H. and L. H. 1:80.

Four later determinations with middle milk were made 11, 14, 20, and 25 days after the second injection. In the injected quarters the titers fluctuated between 1:640 and 1:320. In the other quarters the titer gradually rose to 1:320 on the 14th day in R. H., which then fluctuated between 1:160 and 1:320. L. H. reached a titer of 1:320 on the 20th day and remained there at the next test. The titer of the blood was 1:5,120 on the 14th day and continued there during the remaining tests.

The hemolytic streptococci again disappeared from the plates from the injected left fore quarter on the 4th day and did not return during the remaining tests although they were present in abundance in the other quarters.

On Jan. 26, two guinea pigs received into the peritoneal cavity each 5 cc. of sedimented and centrifuged milk from *R. F.* Neither guinea pig showed any lesions after 3 weeks and cultures from the spleen were negative.

The cow was killed Feb. 23, and the udder reserved for gross and microscopic examination. The only tuberculosis lesion found in this positively reacting cow was in the left bronchial lymph node. The focus was the size of a walnut and partly calcified. The udder presented no lesions recognizable with the

unaided eye and palpation after the organ had been cut into thin slabs. Sections of fixed and hardened tissue from both inoculated fore quarters and the right hind quarter as a control were examined. In all certain lesions were present. Acini, singly and in smaller and larger groups, were filled loosely with polymorphonuclear cells and some acidophils. Each cell of the secreting epithelium in these foci has one large vacuole (fat). The interstitial tissue shows slight infiltrations with lymphoid and plasma cells.

In one section of the left fore quarter there is a focus, occupying two-thirds of a lobule, in which the acini are compressed by endothelial cell accumulations resembling lesions in the guinea pig due to *B. abortus*. The surrounding acini show a modified vacuolated epithelium. The presence of hemolytic streptococci in this udder renders it impossible to ascribe definitely the diffuse lesions to either *B. abortus* or streptococci.

The details of this experiment may be briefly summarized. The injection of living bacilli into the right fore quarter produced a very prompt reaction characterized by febrile disturbances lasting about 2 days and a mastitis shown by marked swelling, heat, and tenderness of the injected quarter, and changes in the physical appearance of the secretion. The swelling lasted a number of weeks.

The agglutinins rose in the injected quarter slightly after 3 days and markedly after 10 days. They rose slightly in all the other quarters after 10 days. The blood was high on the 13th day (not examined earlier).

A second injection into the left fore quarter produced in an accelerated manner all the above phenomena. The agglutinins rose not only in the injected quarter but equally in the right fore, previously injected, and somewhat in the two remaining quarters within 2 days. The blood titer continued to rise and remained high throughout. Injection of milk into guinea pigs failed to demonstrate living bacilli in the inoculated quarters.

Incidentally, the hemolytic streptococci parasitic in the udder disappeared for a time after each injection from the inoculated quarter.

The tentative inference to be drawn from this experiment was a local production of agglutinins in the inoculated quarters. The appearance in the other quarters and in the blood may be ascribed to the dissemination and multiplication of the bacilli both locally in the udder and generally in the system.

II. The Effect of Cultures of Bacillus abortus Killed by Heat after Injection First under the Skin and into a Vein and Then into the Udder.

The first experiment did not prove clear-cut owing to the opportunity for the dissemination of the living bacilli. An experiment with killed cultures had been going on at the same time. It was first attempted to define the relation of the milk to the blood agglutinins by treating a cow with subcutaneous injections to note the effect on the udder agglutinins and then inject heated bacilli into the udder directly.

Cow 487.—Gave birth to a full-term, female calf, slightly under weight, on Mar. 30, 1920. The placenta was discharged *in toto*. There were no indications of disease except for the presence of scattering villi in the cotyledons which were tipped with whitish calcareous incrustations. The cow had reacted to tuberculin.

The blood agglutinins had been tested before the animal was used for the experiment on the following dates and with agglutinin limits as indicated: Nov. 25, 1919, 1:10+, Jan. 6, 1920, 1:20, Jan. 30 and Feb. 25, 1:80. On the day of calving (Mar. 30) the serum titer was 1:160, the colostrum titer 1:320. The serum titer was found to be 1:40 on Oct. 13 and 22. The titer of the mixed milk on the day following parturition was only 1:20. The same titer was found July 13 and Oct. 22.

On Nov. 4, 1920, the cow received subcutaneously 10 cc. of a suspension of *B. abortus* killed by heat, equivalent to 30 cc. of standard turbidity. The strain of *B. abortus* was the same as that used in Experiment I.

The vaccine was prepared by growing the culture on standard agar in a Blake bottle, sealed with sealing wax and incubated at 37°C. for about 72 hours. The growth was washed off in 0.85 per cent NaCl solution and the density reduced by means of the Gates instrument to 2.4. The bacilli were killed in a sealed tube immersed in a water bath at 62°C. for 25 minutes. The heated suspension was incubated over night to allow for any multiplication of surviving bacilli, then two agar slants were inoculated with 2 drops of the suspension, sealed, and incubated to test the sterility of the vaccine.

In the course of the following 6 days, a slight swelling appeared locally and the temperature rose to 39.1°C. and fell again. On Nov. 10, the blood titer had risen to 1:320. The titer of the milk of the four quarters was 1:10. On Nov. 14, the titer of milk from the right quarters was 1:10, from the left 1:20. On Nov. 17, 12 days after the injection, the blood titer was up to 1:640, the milk titer back to 1:10. On Nov. 29, the blood was somewhat below 1:640, the milk titer remained the same.

A second subcutaneous injection of heated bacilli was given Dec. 4, on the opposite (right) side of the neck. The dose was the same as the first.

During the next 5 days, the local swelling appeared a trifle larger and more sensitive than that following the first dose. On Dec. 9, the blood titer was 1:320, the milk titer also a trifle lower than heretofore. Possibly the cow was passing through a slight "negative" phase. On Dec. 16, the 12th day after the second injection, the blood titer had risen to nearly 1:1,280, the milk titer was again 1:20.

On Dec. 20, both titers were as on Dec. 16. On this day a third injection of heated bacilli was given on the right side of the neck, anterior to the site of the first injection. The dose was 8 cc. of a six times normal density. The local swelling this time reached the size of a large hen's egg and remained so for several weeks. 3 days after the injection a conjunctivitis of the right eye appeared with clouding of cornea which cleared up after several weeks.

On Dec. 27, the blood titer was 1:1,280, that of the milk was a trifle above 1:20. On Dec. 31, the milk titer was 1:40. On Jan. 6, the milk titer was 1:40 and the blood titer 1:1,280. On this day, 17 days after the last injection, a dose of 5 cc. of a triple standard suspension of heated bacilli was injected into a jugular vein. Within 2 hours the respirations had increased in number. Within 4 hours the animal had a severe chill. The temperature rose to 41.2°C. On the following day the cow refused part of her food and the milk secretion was reduced. Next day conditions had returned to normal. On Jan. 14, 8 days after the intravenous dose, the blood titer had risen to 1:2,560. The fore milk was 1:20, the middle and last milk 1:40.

The various injections made thus far had produced a relatively high agglutinin titer of the blood serum, even higher than is usually found in infected cows. However, the titer of the milk had risen only very slightly.

On Jan. 21, 18 cc. of a standard suspension of *B. abortus*, killed by heat, were injected directly into the duct of the right fore quarter of the udder. Within 12 hours the cow became quite sick in appearance. The respirations were rapid and the milk secretion reduced one-fourth. The injected quarter was swollen and warm, the secretion thicker than normal and diminished in quantity. On Jan. 23, the injected quarter was still swollen. The agglutinins in the middle milk from two quarters were increased: *R. F.* 1:160, R. H. 1:80, L. F. and L. H. 1:40. On Jan. 24, the injected quarter was still swollen and warm, the fore milk thick and yellowish. Milk titers the same as yesterday. On Jan. 26, the fore milk of the injected quarter had cleared up but the quarter was still a little swollen. The milk titers had dropped slightly.

On Jan. 28, the titers were as follows: *R. F.* 1:160, the rest 1:40. On Jan. 31, 10 days after the injection into the udder, the milk titer of the injected quarter was still 1:160, that of the three other quarters had risen to 1:80. The blood titer was 1:2,560. On Feb. 2, the titer of *R. F.* milk was up to 1:320, that of the rest about 1:80. On Feb. 7, the titer of the *R. F.* milk was still higher, 1:640, and the others had risen to 1:320.

On Feb. 10, the milk titer was unchanged. The blood titer was not 1:5,120. On Feb. 11, the titer of *R. F.* was still 1:640. On this day five guinea pigs received into the peritoneal cavity each about 5 cc. of milk sedimented and centrifuged

from 1 liter taken from the right fore quarter. None showed any enlarged spleen after 6 weeks, nor were cultures positive for *B. abortus*. The test was to determine whether any living bacilli had invaded the udder. On Feb. 16, there was a slight decline in the milk titers. The *R. F.* titer was 1:320, the rest 1:160.

As late as Feb. 21, 31 days after the injection into the right fore quarter, this part seemed a little large and the amount of milk secreted below that of the pre-inoculation period. On this day the milk titers were as before.

The cow was killed Feb. 24. The autopsy showed five nodes in the dorsal mediastinal chain containing caseous and calcareous foci. The lymph nodes were up to a walnut in size. The udder was obtained and thoroughly dissected. Macroscopic lesions were not detected. In two of four sections of *R. F.* there were several areas in which five to six contiguous acini were filled with polynuclear cells. In the interstitial tissue occasional foci of lymphocytes.

The experiment may be summarized as follows: A cow received three subcutaneous and one intravenous injection of a heated suspension of *Bacillus abortus*. As a result the agglutinins of the blood rose from 1:40 to 1:2,560 in 71 days, the titer of the milk from 1:10 to a feeble reaction at 1:40.

On the 78th day, a heated suspension of *Bacillus abortus* was injected directly into the cistern of the right fore quarter of the udder. The agglutinins rose within 2 days in the injected quarter and after 10 days in the others. The titer slowly rose in the injected quarter to 1:640 where it remained until the 20th day when it fell slightly. A similar but less pronounced curve was followed in the other quarters, the maximum being 1:320.

The repeated injection of killed bacilli thus led to a concentration in the blood serum of agglutinins as high as or even higher than that which appears in the spontaneous disease.

The udder agglutinins rose but very little as a result of this preliminary treatment. On the other hand, the direct injection of killed bacilli into the ducts of one quarter of the udder led to a considerable rise in the agglutinins of the milk of that quarter and to a moderate rise in the other quarters.

III. The Direct Exclusive Injection of Dead Bacilli into the Udder Ducts.

In the four following cases the heated bacilli were injected into the udder ducts without any preliminary injection into the subcutis or veins.

No. 584.—Eastern cow, purchased Mar. 22, 1921. Second or third calf born Jan. 5, 1921. Three guinea pigs which received sedimented and centrifuged milk from the udder just before the first injection were negative as to *B. abortus*.

The experiment comprised two injections of 16 cc. each of a standard suspension of heated bacilli (3,300 million per cc.), the first into the right fore quarter, the second into the right hind quarter, 31 days after the first.

There was the usual effect on the udder and its secretion. The injected quarter became swollen to nearly twice its normal size within 18 hours and slowly shrank but it did not regain its original volume in 4 months. After the second injection, the right hind quarter became enlarged in the same way. The fore milk after the first injection was thick and viscid for 5 days. The total milk secretion fell from about 18 to 14 lbs. in 3 days. On the 10th day it was again 18 lbs. The secretion after the injection of the right hind quarter fell from 18½ to 13¾ lbs. in 3 days. On the 6th day it was 16¾ lbs. A slight febrile reaction accompanied each injection and lasted about 24 hours.

The changed physical condition of the milk following the injection was associated with a marked cellular emigration as was the case in the preceding experiments. Following the second injection a count of the leucocytes was carried out. In Table II the number of cells per cubic centimeter and the corresponding limit of the agglutinin titer are given. It will be noted that the maximum cell output occurred within 24 hours after the injection, whereas the agglutinin titer did not rise until the 10th day when the cell content had fallen to the pre-inoculation level. The cell content of *R. F.*, L. F., and L. H. before the second injection was 2 million, 20 million, and 12 million per cc. respectively.

The agglutinin content of the milk and blood serum during the period of observation is best shown in Table III.

It will be noted that, following the first injection, the agglutinins of the affected (*R.F.*) quarter rose slightly on the 2nd and again on the 8th day and on the 29th day to a maximum of 1:320. The L. F. and the L. H. also showed increases slightly above what should be expected as coming from the blood. The R. H. remained unaffected. The blood in the meantime rose to a slightly higher level (1:640).

After the second injection the affected (*R. H.*) quarter developed a relatively high agglutinin content with a maximum of 1:640 on the 19th and 21st days. At the same time, the quarter injected first (*R. F.*) not only continued its high content of 1:320 but rose to 1:640 on several occasions. A relatively high agglutinin output (1:320) was found as late as September 15, nearly 4½ months after the second injection. On the other hand, the two "control" quarters remained fairly low (1:40) throughout. The blood titer at this date had dropped to 1:160.

The bacterial flora was examined before the first injection of the udder. A hemolytic staphylococcus was the chief species. At times other types were present. The marked difference in numbers of colonies on blood agar plates in fore, middle, and last milk makes the meager examinations yield inconclusive results and the figures are therefore omitted.

TABLE II.

Cow 584. Cellular Reaction Following Second Inoculation into the Udder.

Date.	R. H.	
	Agglutination titer.	Cell count.
1921		
May 2	+1:10	637,645
May 6	15 cc. of dead bacilli injected.	
May 7	±1:40	61,543,361
" 8	±1:40	47,193,796
" 9	±1:40	15,306,096
" 10	+1:20	3,826,524
" 12	+1:40	1,913,262
" 16	±1:320	656,631
" 20	±1:320	
" 25	+1:640	1,272,508
Sept. 8	+1:320	3,507,647

Cow 600.—Brought from the Middle West Nov. 20, 1920. Reacted to tuberculin. Received at the Department May 2, 1921. Milk tested on two guinea pigs for *B. abortus* with negative outcome.

On June 7, a standard suspension of a culture of *B. abortus*, killed by heat, was prepared and 15 cc. injected into the ducts of *R. F.* The effect on the udder was as described in the preceding cases. The fore milk was thick and viscid or "stringy." The total amount fell from 25¾ to 22½ lbs. on the 3rd day. On the 23rd day the total yield had risen to 25½ lbs.

On June 30, a second injection of the same dose into *L. F.* was followed by a much greater swelling of the treated quarter. The fore milk was very thick, tending to obstruct the teat canal. The total yield fell again from 25½ to 19 lbs. on the 2nd day. It then began to rise until on the 7th day it was 24⅜ lbs. Each injection was followed by a rise in body temperature to between 40° and 41°C. which lasted about 24 hours.

The changes in the agglutinins of the milk and blood are given in Table IV. Only middle milk was tested.

The reaction to the first injection was less pronounced than in former cases. The agglutinins in the treated quarter rose from less than 1:10 to 1:20 in 8 days and to 1:40 in 20 days. The other three quarters responded by a final rise from less than 1:10 to 1:20 in 20 days. The blood rose from 1:80 to 1:640 in the same interval.

After the second injection into the *L. F.*, the agglutinins in this quarter rose from 1:20 to 1:320 in 12 days, then fell slowly. $2\frac{1}{2}$ months after this injection the milk titer was still 1:80. At the same time the agglutinins in the quarter first injected (*R. F.*) began to rise from 1:40 and reached a maximum of 1:160 in 6 days. Here they remained for 20 days. $2\frac{1}{2}$ months after the second injection the milk from this quarter was still 1:40. One of the two untreated quarters reacted slightly to the second injection by a rise in the agglutinin titer from 1:20 to 1:40. The titer of the other quarter remained as it was after the first injection.

After the second injection the blood titer rose again from 1:320 to a peak of 1:1,280 in 19 days. It remained at or above 1:640 from the 9th to the 27th day after the injection when the tests were discontinued. After a period of $2\frac{1}{2}$ months from the second injection the blood titer stood at 1:160.

The cellular reaction following the injections which partly accounts for the thick, stringy fore milk closely resembled that observed in No. 584 and is shown in Table V. On the day following the first injection the fore milk contained a heavy deposit after sedimentation which consisted of cells, chiefly polynuclear. Only a few mononuclears were present, together with a blue-staining amorphous substance holding cells together. On the 2nd day a sample of middle milk had a heavy floating layer, made up chiefly of cellular elements. On the 3rd day a definite cell sediment or floating layer had disappeared.

Cow 601.—Introduced from the Middle West Nov., 1920. Giving milk at this time. Reacted positively to tuberculin and obtained for the experiment May 2, 1921. Two guinea pigs receiving 5 cc. of sedimented and centrifuged milk the day before the first injection were negative as to *B. abortus*.

Two injections of 15 cc. of a standard suspension of *B. abortus* killed by heat were made, one on June 29 into *R. F.*, and one on July 18 into *R. H.* There

Cow 584. Injection of D

Agg

Date.	Milk.														
	R. F.							R. H.							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10
1921 Apr. 5	16 cc. of dead *B. abortus* culture injected into right fore quarter. (Mixed milk 1:10.)														
Apr. 6	+	+	±	−	−	−		±	−	−	−	−			+
" 7	++	+	+	±	−			+	+	−	−	−			
" 8	++	+	±	−	−			+	+	−	−	−			+
" 9	+	+	±	−	−			+	±	−	−	−			+
" 11	+++	++	+	+	−	−		++	+	±	−	−			++
" 13	++++	++	+	+	±	−	−	++	+	±	−	−			++++
" 15	+++	++	+	+	−	−	−	++	+	−	−	−	−		C.
" 18 / " 19	++	+	+	−	−	−	−	+	±	−	−	−	−		++++
" 21	++	+	+	−	−	−	−	+	−	−	−	−	−		+++
" 25	++	++	+	−	−	−	−	+	−	−	−	−	−		++
May 2	++++	++++	+++	++	+			+	−	−	−	−	−		++++
" 4	++++	++++	++++	+++	++	+	−	+	−	−	−	−	−		++++
May 6	16 cc. of dead *B. abortus* culture injected into right hind quarter.														
May 7	C.	++++	++++	+++	++	+	±	++	+	±	−	−	−	−	+++
" 8	"	C.	++++	++++	+++	++	±	++	+	±	−	−	−	−	++++
" 9	"	++++	++++	++++	+++	++	+	++	+	±	−	−	−	−	+++
" 10	++++	++++	++++	++++	+++	++	+	++	+	−	−	−	−	−	++
" 12 / " 13	++++	++++	++++	++++	+++	+	−	++	++	+	−	−	−	−	+++
" 16	C.	C.	+++	+++	+++	++	+	++++	++++	++	++	+	±	−	+++
" 20	"	++++	++++	+++	++	+	±	C.	++++	++++	++	+	±	−	+++
" 23	++++	++++	++++	+++	++	+	−	++++	++++	+++	+++	++	+	−	+++
" 25		C.	++++	+++	++	+	−		++++	++++	+++	++	++	+	+++
June 1	C.	"	++++	+++	++	±	−	++++	++++	++++	+++	++	+	−	++
" 13	"	"	++++	+++	++	++	±	C.	++++	+++	+++	++	++	±	++
" 27	"	"	++++	++++	++	++	±	++++	++++	++++	+++	+++	++	+	++
" 30															
July 12	C.	C.	C.	++++	+++	++	+	C.	C.	C.	++++	+++	++	+	++
" 26	"	"	++++	++	+	−	−	"	"	"	++++	+++	+	−	
Sept. 8										"	"	++++	++	+	−
" 15	C.	C	++++	++	+	−	−	C.	"	++++	+++	+	±	−	++
" 17															

In the tables C. indicates complete agglutination and clearing of the fluid.

TABLE III.

Cow 584. Injection of Dead Bacilli into the Udder Ducts.

Agglutination Tests.

Milk.

H.				L. F.						L. H.					
1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	
milk 1:10.)															
−	−			+	−	−	−	−		+	−	−	−	−	
−	−									++	+	−	−	−	
−	−			+	±	−	−	−		+	+	−	−	−	
−	−			+	+	−	−	−		+	+	±	−	−	
−	−			++	+	+	±			++	+	+	±	−	
−	−			++++	+++	++	+	±	−	+++	+++	++	+	−	
−	−	−		C.	++++	+++	++	+	−	++++	+++	++	+	+	
−	−	−		++++	+++	++	+	−	−	+++	++	+	+	−	
−	−	−		+++	++	+	−	−	−	++	+	±	−	−	
−	−	−		++	+	+	−	−	−	++	+	+	−	−	
−	−	−		++++	+++	++	+	+	−	++++	+++	++	+	−	
−	−	−		++++	+++	++	+	±	−	+++	++	++	+	±	
−	−	−	−	+++	+++	++	+	−	−	++++	+++	++	+	±	
−	−	−	−	++++	+++	++	+	−	−	++++	+++	++	+	−	
−	−	−	−	+++	++	+	±	−	−	+++	++	+	±	−	
−	−	−	−	++	+	±	−	−	−	++	++	+	±	−	
−	−	−	−	+++	++	+	−	−	−	+++	++	+	−	−	
++	+	±	−	+++	++	+	−	−	−	+++	++	+	±	−	
++	+	±	−	+++	++	+	−	−	−	+++	++	+	−	−	
++	++	+	−	+++	++	+	±	−	−	+++	++	+	−	−	
++	++	++	+	+++	++	+	−	−	−	++++	++	+	−	−	
++	++	+	−	++	++	+	−	−	−	+++	++	+	−	−	
++	++	++	±	++	+	±	−	−	−	++	+	±	−	−	
++	+++	++	+	++	+	±	−	−	−	+++	++	+	±	−	
+++	+++	++	+	++	+	−	−	−	−	++	+	−	−	−	
+++	+++	+	−												
+++	++	+	−												
++	+	±	−	++	−	−	−	−	−	+	−	−	−	−	

	Blood.							
:320	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
	++++	++	++	+	+	±	–	
	C.	C.	C.	++++	++	+	+	–
	C.	C.	C.	C.	C.	+++	++	–
	"	"	"	"	"	+++	+	–
	C.	C.	C.	C.	C.	+++	+	–
		C.	C.	C.	++++	++	–	–
		C.	C.	C.	++++	+	–	–
		"	"	"	C.	+++	+	–
		"	"	"	"	++	+	–
		C.	C.	C.	C.	++++	+	–
		"	"	"	++++	±	–	–
		"	"	"	++++	±	–	–
		C.	C.	C.	+++	–	–	–
		"	"	"	++++	–	–	–
		"	"	++++	+	–	–	–
		C.	C.	C.	++	±	–	–

TABLE IV.

Cow 600. Injection of Dead Bacilli into the Udder Ducts

Agglutination Tests.

Date.	Milk.															
	R. F.					R. H.			L. F.						L. H.	
	1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20
1921																
May 25	−	−	−	−	−	−	−	−	−		−	−	−	−		−
June 7	15 cc. of dead bacilli injected into right fore quarter.															
June 8	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 9	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 10	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 11	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 13	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 15	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 17	++	+	−	−	−	±	−	−	−	−	−	−	−	−	−	−
" 21	++	+	−	−	−	+	±	−	+	+	−	−	−	−	+	±
" 27	+++	++	+	−	−	++	+	−	++	±	−	−	−	−	++	+
June 29	15 cc. of dead bacilli injected into left fore quarter.															
June 30	+++	+++	++	+	−	++	+	−	+++	+	−	−	−	−	++	++
July 1	+++	++	+	±	−	++	+	−	+++	++	++	+	−	−	++	++
" 2	+++	++	++	+	−	++	+	−	+++	+++	++	+	+	−	+++	++
" 6	+++	++++	+++	++	+	++	+	−	++++	++++	++++	++	+	−	+++	+++
" 8	++++	+++	+++	++	+	++	+	−	++++	++++	++++	+++	++	−	+++	++
" 11	++++	++++	+++	++	+	+++	++	+	C.	C.	C.	++++	++	+	+++	++
" 18	++++	+++	++	+	+	+++	++	±	"	"	"	+++	++	±	+++	+++
" 26	++++	++++	+++	++	±	+++	+	−	++++	++++	+++	++	+	−	++++	++
Sept. 15	++	++	+	−	−	−	−	−	+++	++	++	+	−	−	−	−
" 17																

TABLE IV.

Dead Bacilli into the Udder Ducts.

lutination Tests.

F.				L. H.				Blood.						
	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:20	1:40	1:80	1:160	1:320	1:640	1
	–	–	–		–	–	–	++++	++	+	–	–	–	
	–	–	–	–	–	–	–	++++	++	–	–	–	–	
	–	–	–	–	–	–	–	C.	++	–	–	–	–	
	–	–	–	–	–	–	–	"	++	–	–	–	–	
	–	–	–	–	–	–	–	"	++++	+	–	–	–	
	–	–	–	–	–	–	–	"	C.	++++	+	–	–	
	–	–	–	–	–	–	–	"	"	+++	++	+	–	
	–	–	–	+	±	–	–	"	"	C.	+++	++	+	
	–	–	–	++	+	±	–							
								C.	C.	C.	++++	++	–	
	–	–	–	++	++	+	–							
	+	–	–	++	++	+	–							
	+	+	–	+++	++	+	±	C.	C.	C.	++++	++	–	
++	++	+	–	+++	+++	++	+	"	"	"	+++	+	–	
++	+++	++	–	+++	++	+	±	"	"	"	++++	++	+	
	++++	++	+	+++	++	+	–	"	"	"	++++	+++	++	
	+++	++	±	+++	+++	+	–	"	"	"	C.	C.	+++	
+	++	+	–	++++	++	+	–	"	"	"	"	++++	++	
-	+	–	–	–	–	–	–							
								C.	C.	++++	±	–	–	

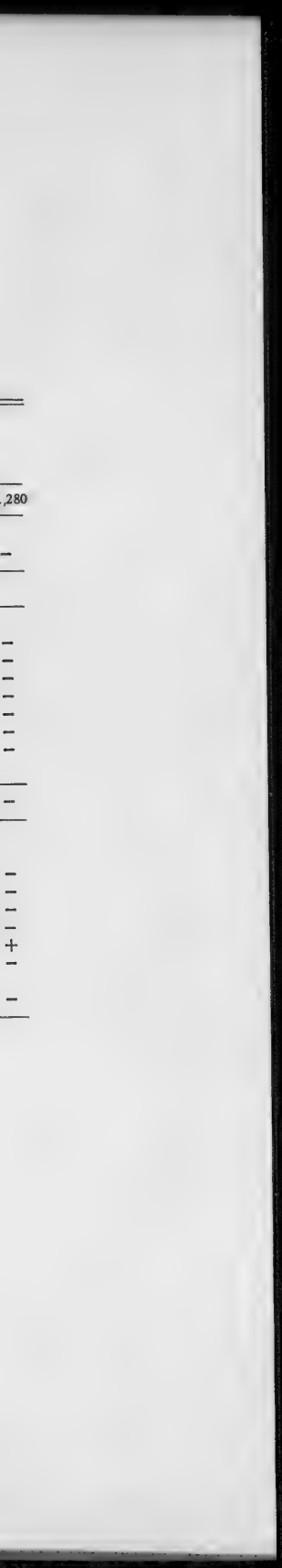

TABLE V.

Cow 600. Cell Counts and Agglutinin Limits in the Milk.

Date.	R. F.		R. H.		L. F.		L. H.	
	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.
1921								
May 25	—	956,631	—	4,145,401	—	319,127	—	798,449
June 7	15 cc. of dead bacilli injected into right fore quarter.							
June 8	±1:10	182,077,767	—	5,102,032	—	318,877	—	6,377,540
" 9	+1:10	83,545,774	—		—		—	
" 10	+1:10	8,928,556	—		—		—	
" 11	±1:10	4,145,401	—		—	956,631	—	
" 13	±1:10	2,869,893	—		—		—	
" 15	+1:20	31,887,700	—		—		—	
" 17	+1:20	3,188,770	±1:10		—		—	
" 21	+1:20	5,739,789	±1:20		+1:20		±1:20	
June 29	15 cc. of dead bacilli injected into left fore quarter.							
June 30	+1:80	21,045,882	+1:20	8,709,679	+1:20	122,129,891	+1:40	6,377,540
July 1	±1:80		+1:20		+1:80	107,461,549	+1:40	
" 2	+1:80		+1:20		+1:160	90,240,191	±1:80	
" 6	+1:160		+1:20		+1:160	6,058,663	+1:80	
" 8	±1:320		+1:20		++1:160	5,739,786	±1:80	
" 11	±1:320		+1:40		+1:320	1,913,262	+1:40	
" 26	±1:160	22,959,144	+1:20		+1:160	637,754	+1:40	

was the same febrile reaction and prompt swelling of the injected quarters already described. The milk was thick both times, but cleared up on the 4th day. The drop in the total daily milk output occurred each time with nearly complete restitution within a week. The agglutinin titer of the milk from the four quarters changed but little following the first injection. That of *R. F.* rose from less than 1:10 to 1:20. The same took place in L. H. The other quarters rose to a bare 1:10. The blood titer, however, rose in 15 days from 1:10 to 1:320.

After the second injection a similar inertia in the local and general production of agglutinins was observed. *R. H.* rose on the 8th day to 1:20. *R. F.* rose one step to 1:40 after the 8th day. The other quarters remained unchanged. The blood titer also did not rise higher.

The cellular reaction following each injection was prompt and pronounced, as shown in Table VI. The limiting agglutinin titer is also given. Both agglutinin determination and cell count were made on the same sample of middle milk collected as nearly as ascertainable after the quarter had been half emptied.

At the time of the first injection the L. H. (untreated) was in a state of inflammation as shown by the very high content of cells. Traumatism due to a fall was suspected as the cause, but no definite proof of this is forthcoming.

A bacteriological examination of the fore milk before the udder injection on May 25 gave the following result.

R. F. 1,190 colonies per cc. of hemolytic streptococci.
R. H. 70 colonies per cc. of staphylococci.
L. F. 140 colonies per cc. of staphylococci.
L H. 670 colonies per cc. of staphylococci.

On June 30, the day following the injection of *R. F.*, the bacteria content of the middle milk was as follows:

R. F. 20 colonies per cc. of hemolytic streptococci.
R. H. 440 colonies per cc. of staphylococci.
L. F. 900 colonies per cc. of both species.
L. H. 300 colonies per cc. of both species.

On July 6, 7 days after the injection, the bacteria content of the middle milk was as follows:

R. F. 600 colonies per cc. of both species.
L. H. Countless colonies chiefly staphylococci.

No further examinations of the udder flora were made. 92 days after the second injection a spontaneous mastitis appeared in the *R. H.* with a high cell count, 38 million, and a low bacterial count of 10 colonies per cc.

Cow 897.—Grade Holstein. Calved shortly before being shipped from the Middle West. Received Dec. 14, 1921. Cow weighs 990 lbs. Reacted to the tuberculin test Feb., 1922.

TABLE VI.

Cow 601. Cell Counts and Agglutinin Limits in the Milk.

Date.	R. F.		R. H.		L. F.		L. H.	
	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.
1921								
May 25	—	7,105,294	—	637,754	—	478,315	—	857,112
June 29	15 cc. of dead bacilli injected into right fore quarter.							
June 30	—	132,652,832	—	637,754	—	2,232,129	—	119,259,998
July 1	±1:10	43,367,272	—		—		—	69,196,309
" 2	—	11,160,695	±		—		—	15,306,096
" 6	±1:20	35,714,224	—		—		±1:10	2,869,893
" 8	+1:20	10,204,064	—		+1:10		±1:20	16,262,727
" 11	+1:20	39,859,625	±1:10		+1:10		+1:20	17,857,112
" 18, a.m.	+1:10	12,436,263	—	956,631	—	956,631	+1:10	14,987,219
July 18, p.m.	15 cc. of dead bacilli injected into right hind quarter.							
July 19	+1:10	10,204,064	+1:10	73,660,557	—	1,275,508	+1:10	11,479,572
" 20	+1:10		+1:10	100,127,378	—	637,754	±1:10	
" 22	+1:20	35,395,347	+1:10	14,030,588	—	956,631	+1:10	47,831,550
" 26	+1:20	12,117,326	+1:20	3,188,770	+1:10	2,232,139	+1:20	12,117,326

In March a preliminary bacteriological study of the udder secretion was made. A variety of bacteria were found in the milk, among them streptococci, staphylococci, and diphtheroid forms. The limiting agglutinin titer of the milk toward these types varied from 1:10 to 1:160. It was assumed as heretofore that these forms would have no direct influence on the production of *B. abortus* agglutinin. This at the outset was less than 1:10. This experiment differed from the preceding in that repeated injections of dead bacilli were made into the same quarter. The suspension was of the density used heretofore and the amount introduced through a milking tube about 20 cc. each time. The agglutinin titers of milk and blood are given in Table VII.

It will be noticed that the agglutinin production may be considered medium when compared with Nos. 584 and 487. The response of the udder, slow and slight after the first injection, is accelerated and quantitatively increased after the second injection. After the third injection the titer of the milk is a trifle above that of the blood. The response of the other three quarters is slight and perhaps largely due to the rise in blood agglutinins.

The leucocytic reaction appeared as heretofore in the next morning's milk and reached a maximum then. Following the first injection the sedimented mass of cells in next morning's milk was equivalent to 8 per cent of the volume of milk of the injected quarter (887 cc.). On the following day the cell mass was very slight. Only a few flakes were detected on the 3rd day. The same reaction followed the second and the third injection. The cellular sediment disappeared after the 2nd day. The relatively high percentage of cellular sediment amounting to 8 per cent of the total milk secretion is in part due to the large amount of fat taken in by the leucocytes which may represent one-half the total volume of the leucocytic deposit.

After the third injection of heated bacilli into *R. F.* two injections of normal salt solution were made into L. F. 12 days apart. A slight increase in specific agglutinins was detected in the milk. The agglutinin content of *R. F.* maintained its original high titer as shown in Table VII. No appreciable cellular reaction followed the injection of salt solution.

TABLE VII.

Cow 897. Injection of Dead Bacilli into the Udder Duc

Agglutination Tests.

Date.	Milk.															
	R. F.						R. H.				L. F.				L.	
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:10	1:20
1922																
Mar. 20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mar. 23	20 cc. of dead bacilli injected into right fore quarter.															
Mar. 24	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 27	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 29	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 31	+++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Apr. 3	++++	++	+	−	−	−	±	−	−	−	−	−	−	−	−	−
" 7	++	+	−	−	−	−	−	−	−	−	+	±	−	−	−	−
" 11	++	+	−	−	−	−	+	−	−	−	+	±	−	−	±	−
Apr. 17	20 cc. of dead bacilli injected into right fore quarter.															
Apr. 18	++	+	−	−	−	−	+	−	−	−	+	−	−	−	+	−
" 19	++	+	−	−	−	−	+	−	−	−	+	−	−	−	+	−
" 21	++++	++++	++++	++	+	−	+	−	−	−	+	−	−	−	+	−
" 22																
" 25	C.	C.	C.	++++	+++	+	++	+	+	±	++	+	+	±	++	+
" 28	"	"	++++	++	+	−	++	+	−	−	++	+	−	−	++	+
May 1	"	"	++++	+	±	−	+	±	−	−	+	±	−	−	+	±
" 4	"	"	+++	++	+	−	+	±	−	−	+	±	−	−	+	±
" 9	"	++++	+++	++	+	−	+	−	−	−	+	±	−	−	+	−
May 11	20 cc. of dead bacilli injected into right fore quarter.															
May 12	C.	C.	+++	+	−	−	+	−	−	−	+	±	−	−	+	−
" 13	"	"	++++	++	+	−	+	±	−	−	+	±	−	−	+	−
" 15		"	C.	+++	+	±	+	±	−	−	+	−	−	−	+	−
" 17	C.	"	"	++++	+++	+	+	±	−	−	+	±	−	−	−	−
" 22	"	"	"	C.	+++	+	++	+	+	−	++	+	±	−	++	+
" 29	"	"	++++	++++	++	±	++	±	−	−	++	+	−	−	++	+
May 31	20 cc. of normal salt solution injected into left fore quarter.															
June 2	C.	C.	++++	+++	++	−					++	+	−	−	+	±
" 5	"	"	++++	+++	++	+	++	±	−	−	+++	+	±	−	+	±
" 12	"	"	++++	++	+	±	+	±	−	−	+++	+	±	−	+	±
	20 cc. of normal salt solution injected into left fore quarter.															
June 13	C.	C.	++++	++	+	+	+	+	−	−	+++	+	+	−	+	+
" 14	"	"	++++	+++	++	+	+	±	−	−	++	+	±	−	+	±
" 16							+	−	−	−	+++	++	±	−	+	−
" 23	C.	++++	+++	++	+	−	+	−	−	−	+++	++	+		++	+
" 26	"	++++	+++	++	+	−	+	−	−	−	++	+			±	

TABLE VII.

Cow 897. Injection of Dead Bacilli into the Ud

Agglutination Tests.

Milk.									
	R. H.				L. F.				
1:320	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:
–	–	–	–	–	–	–	–	–	
ht fore quarter.									
–	–	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	–	
–	±	–	–	–	–	–	–	–	
–	–	–	–	–	+	±	–	–	
–	+	–	–	–	+	±	–	–	
t fore quarter.									
–	+	–	–	–	+	–	–	–	
–	+	–	–	–	+	–	–	–	
–	+	–	–	–	+	–	–	–	
+	++	+	+	±	++	+	+	±	+
–	++	+	–	–	++	+	–	–	+
–	+	±	–	–	+	±	–	–	
–	+	±	–	–	+	±	–	–	
–	+	–	–	–	+	±	–	–	
ht fore quarter.									
–	+	–	–	–	+	±	–	–	
–	+	±	–	–	+	±	–	–	
±	+	±	–	–	+	–	–	–	
+	+	±	–	–	+	±	–	–	
+	++	+	+	–	++	+	±	–	
±	++	±	–	–	++	+	–	–	
into left fore quarter.									
–					++	+	–	–	
+	++	±	–	–	+++	+	±	–	
±	+	±	–	–	+++	+	±	–	
into left fore quarter.									
+	+	+	–	–	+++	+	+	–	
+	+	±	–	–	++	+	±	–	
	+	–	–	–	+++	++	±	–	
–	+	–	–	–	+++	++	+		
–	+	–	–	–	++	+			

L. H.				Blood.					
10	1:20	1:40	1:80	1:20	1:40	1:80	1:160	1:320	1:640
–	–	–	–	++	+	±	–	–	–
	–	–	–						
	–	–	–						
	–	–	–	++	±	–	–	–	–
	–	–	–						
	–	–	–	++++	++++	++	+	–	–
	–	–	–	C.	C.	C.	++++	++	±
	–	–	–	"	"	++++	++	+	±
	–	–	–						
	¶	–	–						
	–	–	–						
+	+	+	±	C.	C.	++++	++	+	–
+	+	±	–	C.	C.	C.	++++	++	–
	±	–	–						
	–	–	–						
				C.	C.	C.	+++	+	–
	–	–	–						
	–	–	–	C.	C.	C.	+++	±	–
	–	–	–						
+	+	+	–	C.	C.	C.	++++	+	–
+	+	–	–						
				C.	C.	C.	++++	++	–
	±	–	–						
	±	–	–						
	±	–	–						
	+	–	–						
	±	–	–						
	–	–	–						
	+								

DISCUSSION.

The relation between agglutinins in the blood serum and those in the milk of an actively secreting udder when suspensions of *Bacillus abortus* killed by heat are injected into the subcutis or the circulation directly is much the same as that observed in the spontaneous infection (Experiment II). The agglutinins in the milk occur rarely above 1:40 or below 1:10 and the ratio of content in milk to that in blood fluctuates between 1/32 and 1/64. When the udder is invaded and made the site of multiplication by *Bacillus abortus* then the agglutinins in the milk rise so as to shift the ratio to 1/4, 1/2, or even 1/1 (Experiment I).

So far as is known, the invasion of the udder by *Bacillus abortus* means its presence and multiplication in the milk in the acini and ducts and not in the connective tissue stroma. The condition of the udder tissues within 24 hours after the introduction of dead bacilli into the ducts and later, after subsidence of the acute reaction, indicate this. In other words, the residual milk in the acini and ducts rather than the udder tissue appears to be the seat of multiplication.

When bacilli killed by heat are injected into the udder ducts, there occurs a rise of agglutinins in the quarter receiving the antigen varying in degree with the animal and in rapidity according to former treatment with the same antigen. This rise is accompanied by an increase of specific blood agglutinins usually higher than that of the treated quarter. In fact injection into an udder duct is as effective as subcutaneous or intravenous injection in causing agglutinins to appear in the blood. The rise in agglutinins in the treated quarter may be associated with a slight rise in one or more of the remaining quarters.

If the first injection is followed by a second into another quarter 3 or more weeks later the rise in this quarter is more rapid and more pronounced than that following the first injection. Moreover, there is a simultaneous jump of the agglutinin titer in the quarter which was treated first and there may be a slight additional rise in the two remaining quarters.

These facts are summarized in Table VIII. Only the limiting agglutinin values are given there. The first line of figures under each case represents the maximum titer of each quarter after the first injec-

TABLE VIII.

Summary of Udder Reactions Giving Maximum Output of Agglutinins in the Blood and Milk after Each Injection.

R. F.	R. H.	L. F.	L. H.	Blood.	Daily milk secretion.
Eastern Cow 523. Injection of living bacilli into right fore quarter and left fore quarter.					*lbs.*
1:640	1:320—	1:320—	1:320—	1:1,280	9
1:640	1:320—	**1:640**	1:320	1:2,560	
Native Cow 487. Subcutaneous and intravenous injections of dead bacilli.					
1:40	1:20	1:20+	1:40	1:2,560	8
Injection into right fore quarter.					
1:640	1:320	1:320	1:160	1:2,560	
Eastern Cow 584. Injection of dead bacilli into right fore quarter and right hind quarter.					
1:320	1:40	1:80+	1:80+	1:640	18
1:640	**1:640**	1:40	1:40	1:640	
Western Cow 600. Injection of dead bacilli into right fore quarter and left fore quarter.					
1:40	1:20	1:20	1:20	1:640	25
1:160	1:40	**1:320**	1:80	1:1,280	
Western Cow 601. Injection of dead bacilli into right fore quarter and right hind quarter.					
1:20	1:10—	1:10—	1:10—	1:320	25
1:40	**1:40**—	1:10	1:20—	1:160	
Western Cow 897. Repeated injection of dead bacilli into right fore quarter.					
1:20	1:10	1:10	1:10—	1:320	30
1:160	1:20	1:20	1:20	1:320	
1:320	1:20	1:20+	1:20—	1:320	

tion, or, in the second case, after preliminary injection into subcutis and vein. The second line represents the maximum titer after the second treatment. The bold faced figure represents the titer of the treated quarter. The column next to the last contains the maximum blood titer of the period, the last the daily output of milk at the beginning of the experiment. The milk titer before treatment was either 1:10 or less. Dilutions under 1:10 were not tested.

In evaluating these figures it must be borne in mind that the udders of the experimental cows were in a state of active secretion. The product was not a stored product as is the case with colostrum, in which antibodies tend to accumulate and rapidly disappear when the secretory activity is fully established. There is here a steady loss of agglutinins which is daily replaced over considerable periods of time. This daily loss differed from cow to cow, as shown in the last column of Table VIII. The two first (Nos. 523 and 487) were nearing the end of the lactation period. The others were probably at or near the maximum of productivity.

Of interest as a collateral phenomenon is the "inflammatory" reaction immediately following the injection of dead bacteria into an udder duct. A slight febrile disturbance appears and lasts about 24 hours. The injected quarter becomes swollen, tender, and feels warmer than normal. There is an abundant outpouring of polynuclear leucocytes forming a deposit up to 8 per cent of the total fluid secreted. This reaction is chronologically independent of the rise in agglutinins. Following the first injection the latter appeared fully a week later. Tentatively we may assume the presence of a pyrogenic and an agglutinogenic factor in the killed bacilli.

The material results of the experiments are too meager to warrant any but cautious and tentative interpretations pending further investigation.

The evidence as far as it goes appears to support the hypothesis that after the injection of living or dead bacteria into the udder ducts the increased agglutinin is produced mainly in the udder tissue and that it is not due to an increased permeability of the endothelium or the epithelium of the gland. The increase is localized in the injected quarter and the one previously injected. In Experiment I the general increase in all quarters is probably due to the diffusion of living bacteria into the other quarters. In Experiment II there is

a decided increase in the quarters not injected. The results differ from subsequent ones in this respect. Two explanations suggest themselves. One is the previous treatment of the animal by the subcutaneous and intravenous route. The other is the possibility that some bacilli escaped destruction in the heating of the suspension to be injected. A contributory factor is the low daily output of milk as compared with that of the other cows.

There seems to be no valid reason to bring the rise and persistence of agglutinins in the treated quarter into relation with the prompt high output of polynuclear leucocytes. This appears within 20 hours whereas the rise in agglutinins comes on gradually and is definitely at its maximum in 10 or more days. The tendency of the cellular emigration is rather to sweep out the injected bacteria and thus reduce quantitatively their effectiveness as antigens.

The intimate physical association between udder and blood is demonstrated in several ways in the experiments. The injection into the cistern where the various ducts meet to form the single excretory duct of a suspension of dead bacilli is followed within 20 hours by a febrile reaction and a heavy emigration of polymorphonuclear leucocytes. The injected fluid in no instance was more than 20 cc. and it would hardly be expected to come in contact with the secretory epithelium. The reaction, however, is pronounced.

Another fact which shows the interchange between the blood and the udder is the prompt general response of the body to the antigens by the appearance of agglutinins in the blood in a concentration usually higher than that developed in the treated quarter. This interchange which is very prompt must be carried on through the secreting epithelium of the gland. The injection into the udder ducts is equivalent in its effects to an injection into the trachea, or even into the circulation. These facts indicate an active inward absorptive current probably alternating with active secretion.

CONCLUSIONS.

The foregoing experimental cases point to a distinct participation of the udder in the production of agglutinins when the gland is invaded by living or flooded by dead bacteria. The quarter injected reacts at first with a heavy influx of polynuclear leucocytes and later with an increase of agglutinins.

CHLORIDE RETENTION IN EXPERIMENTAL HYDRONEPHROSIS.

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(Received for publication, August 10, 1922.)

In a previous paper (Keith and Pulford) we described a method for producing experimental hydronephrosis, which permitted the study of renal function in this condition for periods varying from weeks to months. The method used was the partial obstruction of both ureters near the ureteropelvic juncture. After a thorough functional study of hydronephrosis thus produced, further experiments were carried out to ascertain how quickly normal function could be restored after ureteral obstruction was removed. It was found that if the obstruction was removed within a week, renal function was rapidly reestablished, but ureteral obstruction of longer duration often led to permanent damage to the kidney. This method of production of a lesion in the kidney with the later withdrawal of the original cause offered unusual opportunities for observing the excretory function of the kidney during the acute and later reparative stage of a pathologic process. In our previous work the excretion of nitrogenous bodies, particularly urea, and phenolsulfonephthalein was emphasized, whereas at present we are reporting observations on the excretion of chlorides. We have noted in hydronephrosis that the regulating mechanism concerned in excretion of chloride differs in several respects from that controlling the elimination of urea and phenolsulfonephthalein. This finding is in agreement with the results obtained by Widal and Javal, and McLean in other types of renal lesions. In the present study it will be shown that the water and chloride intake must be carefully controlled. Further, it was found that when both urea and chlorides are retained they do not accumulate quantitatively in the same degree in the blood plasma.

Methods.

Female dogs were used exclusively because of the ease of catheterization. Between experiments they were given a diet of 200 gm. of ground beef and 300 cc. of water each day. Each dog was subjected to several control tests before being selected for use. The urine was shown by the routine tests to be free from albumin, casts, and erythrocytes. The excretion of phenolsulfonephthalein and the content of urea in the blood were found to be normal before an experiment was started. The dogs were kept in metabolism cages and in the short experiments they were allowed no food. In the first few experiments, when measured amounts of water were given by mouth as a medium for salt administration, vomiting often occurred and the results were not completely satisfactory. In the later experiments neither food nor water was allowed by mouth and sodium chloride was given subcutaneously, daily, in a concentration of about 1 per cent; that is, approximately 2 gm. to 200 cc. of water, the exact percentage being determined quantitatively in each lot of solution used. Hydronephrosis was produced by applying rubber bands around both ureters (Keith and Pulford).[1] The determinations used in the test of kidney function were: phenolsulfonephthalein excretion by the test of Rowntree and Geraghty, urea of the urine and the whole blood by Marshall's method (1913, *a*, *b*), chlorides of the urine by Arnold's modification of Volhardt's method (the interfering substances that might be present in urine of dogs could scarcely affect the comparative nature of the results), and chlorides of the blood plasma by the method of McLean and Van Slyke. Duplicate determinations were always made.

There is considerable variation in the total amount of urine excreted by a fasting animal under constant fluid intake by mouth. In order that the urine output might be more constant, and that more accurate studies on chloride retention might be possible, a 1 per cent sodium chloride solution was given subcutaneously as noted above. The daily variations in the amount of urine were thereby minimized. Since the urea content of whole blood and plasma differ so slightly, we have considered them here as identical.

[1] All operations were performed under ether anesthesia.

Observations on Control Animals.

Four normal, control, fasting animals on an average daily intake of approximately 2 gm. of sodium chloride in 200 cc. of water given subcutaneously showed, 24 hours later, blood plasma chlorides ranging from 628 to 692 mg. for each 100 cc. If the findings during the

TABLE I.

Normal Controls.

Dog No.	Weight.	Day.	Intake.		Plasma chlorides for each 100 cc.	Urine output.			
			Sodium chloride.	Water.		Volume.	Specific gravity.	Sodium chloride.	
								Total amount.	Amount for each 100 cc.
	kg.		*gm.*	*cc.*	*gm.*	*cc.*		*gm.*	*gm.*
1	10.6	1	1.87	200	650	120	1.050	2.06	1.70
		2	1.87	200	643	98	1.045	1.91	1.95
		3	1.87	200	635	96	1.047	1.79	1.86
		4	1.87	200	650	90	1.050	1.39	1.54
2	6.6	1	2.20	200	672	98	1.040	0.90	0.92
		2	2.20	200	637	92	1.049	1.07	1.16
		3	2.20	200	660	101	1.044	1.55	1.53
		4	2.20	200	660	123	1.038	2.06	1.67
3	7.1	1	2.20	200	685	130	1.045	2.86	2.20
		2	2.20	200	645	201	1.025	2.45	1.22
		3	2.20	200	630	178	1.023	2.24	1.26
		4	2.20	200	628	170	1.023	2.27	1.33
		5	2.20	200	648	145	1.025	2.06	1.42
		6	2.20	200	645	140	1.023	1.97	1.41
4	8.5	1	2.16	200	687	90	1.044	1.87	2.08
		2	2.16	200	675	85	1.045	2.09	2.46
		3	2.16	200	692	78	1.045	1.65	2.14

first control day are omitted, the variations in plasma chlorides in each animal are distinctly small, 635 to 650, 637 to 660, 630 to 648, and 675 to 692 mg. for each 100 cc. respectively. The total urine chlorides varied from 0.90 to 2.86 gm. each day, while the concentration ranged from 0.92 to 2.46 gm. per 100 cc. In one of the control animals (Dog 3) the plasma chlorides were determined at short

intervals during the day, between 10 a.m. and 8 p.m. and the variations found were 628 to 655 mg. (Tables I and II).

TABLE II.

Variation in Plasma Chlorides during the Day.

Dog No.	Weight.	Date and hr.	Intake.		Plasma chlorides for each 100 cc.	Urine output.			
			Sodium chloride.	Water.		Volume.	Specific gravity.	Sodium chloride.	
								Total amount.	Amount for each 100 cc.
	kg.	*1916*	*gm.*	*cc.*	*mg.*	*cc.*		*gm.*	*gm.*
3	7.1	Feb. 5	2.20	200					
		" 6, 10.00 a.m.			646	125	1.025	2.05	1.64
		10.05 "	2.20	200					
		12 m.			655				
		2.00 p.m.			650				
		4.00 "			646				
		8.00 "			628				
		Feb. 7, 10.00 a.m.			645	140	1.023	1.96	1.40

Observations on Animals with Acute Hydronephrosis.

In four animals partial ureteral obstruction was maintained for from 2 to 3 days and then the obstructing bands were removed (Table III). The functional findings in three of these experiments (Dogs 5, 6, and 7) were similar, while in the fourth (Dog 8) the total functional impairment was less marked and the changes in chloride metabolism were distinctly different. Although there was retention of chlorides in each experiment, edema was never observed. During the initial stage of the first three experiments the increase of urea and chlorides in the plasma followed definitely different curves. The greatest chloride increase, from 683 to 729 mg., occurred in Dog 7 while the urea rose from 28 to 156 mg. This relatively slight increase in the chlorides, but marked rise in urea, was accompanied by a definite decrease in the urinary excretion of water, chlorides, urea, and phenolsulfonephthalein. In Dog 6 there was practically a total suppression of urine with no increase in the plasma chlorides, although the urea retention in the blood was marked. This experiment emphasized the fact that when chloride retention occurs in

TABLE III.

Acute Hydronephrosis.

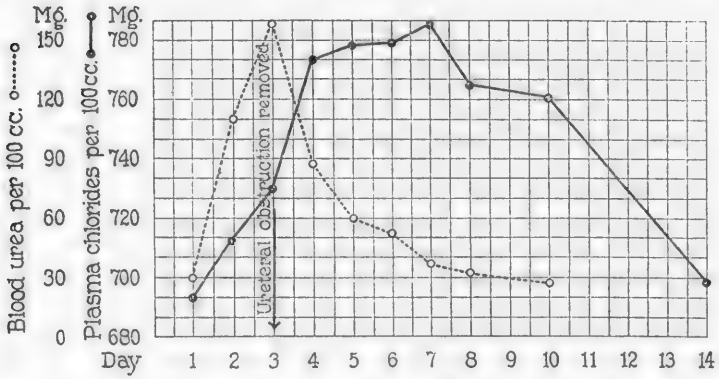
Dog No.	Weight.	Date.	Intake.		Plasma chlorides for each 100 cc.	Blood urea for each 100 cc.	Urine output.				
			Sodium chloride.	Water.			Volume.	Sodium chloride.		Phenolsulfonephthalein excretion for 1 hr.	Urea for each 100 cc.
								Total amount.	Amount for each 100 cc.		
	kg.	*1915*	*gm.*	*cc.*	*mg.*	*mg.*	*cc.*	*gm.*	*gm.*	*per cent*	*gm.*
5*	21.9	Nov. 29	3.00	325		35	151			83	
		" 30	3.00	325	669						
		Dec. 1†	3.00	325							
		" 2	3.00	325							
		" 3‡	3.00	325	664	128				Tr.	
		" 4	3.00	325	730	91	330	0.61	0.18		18.39
		" 5	3.00	325	750	60	230	0.97	0.42	48	13.69
		" 6	3.00	325	718	42	246	1.47	0.59	40	10.29
		" 7	3.00	325	750	40	230	1.57	0.68		10.35
		" 8	3.00	325		40	278	2.45	0.88		7.78
		1916									
6	10.6	Jan. 22	2.14	200		24				66	
		" 24†	2.14	200		34					
		" 25	2.14	200	680	124	3	0	0	0	
		" 26‡	2.14	200	681	222	5	0	0	0	
		" 27	2.14	200	783	79	800	3.20	0.40	43	9.11
		Feb. 28	Dog died of general peritonitis.								
7	19.8	Mar. 10	2.10	200	683	28	226	2.22	0.98	62	
		" 11†	2.10	200	692	28	202	2.21	1.10		11.43
		" 12	2.10	200	712	109	120	0.24	0.20		4.91
		" 13‡	2.10	200	729	156	152	0.22	0.15	8	8.00
		" 14	2.10	200	773	85	415	2.92	0.70		22.31
		" 15	2.10	200	777	60	235	2.77	1.18		15.14
		" 16	2.10	200	778	54	180	1.45	0.80	37	11.68
		" 17	2.10	200	785	37	195	1.17	0.60		11.20
		" 18	2.10	200	765	32	250	2.10	0.84		11.55
	15.7	" 19	2.10	200			260	2.35	0.90		
		" 20	2.10	200	761	27	220	2.30	1.05	57	
		" 24	2.10	200	696						
8	11.2	Feb. 19†	2.18	200	680	21	115	2.07	1.80	64	4.94
		" 20	2.18	200	675	72	112	3.67	3.00		4.27
		" 21	2.18	200	771	66	136	1.52	1.11		6.25
		" 22‡	2.18	200	768	54	202	1.05	0.52	16	
		" 23	2.18	200	751	35	206	3.25	1.57		7.00
		" 24	2.18	200	728	17	140	2.28	1.62	55	4.22
		" 25	2.18	200	716	17	126	1.96	1.55		3.85
		" 26	2.18	200	707	17	145	2.34	1.62	66	4.39
		" 27	2.18	200	700		125	1.93	1.54		
		" 28	2.18	200	685	24	127	2.42	1.57		

* Water and sodium chloride given by mouth.

† Double ureteral bands applied.

‡ Double ureteral bands removed.

renal insufficiency with retention of water, the accumulated chlorides in plasma and tissues may not be made evident by a quantitative chloride estimation in a unit volume of plasma. Dog 6 died of peritonitis before complete restoration to normal function had taken place. With removal of the ureteral obstruction in the first three experiments (Dogs 5, 6, and 7), the plasma chlorides showed a remarkable rise of from 9 to 15 per cent, while the urea decreased rapidly. Corresponding with this elevation of the plasma chlorides, the excretion of water, chlorides, urea, and phenolsulfonephthalein was increased, although the chloride concentration of the urine still remained low (Text-figs. 1 and 2). The urea content of the plasma

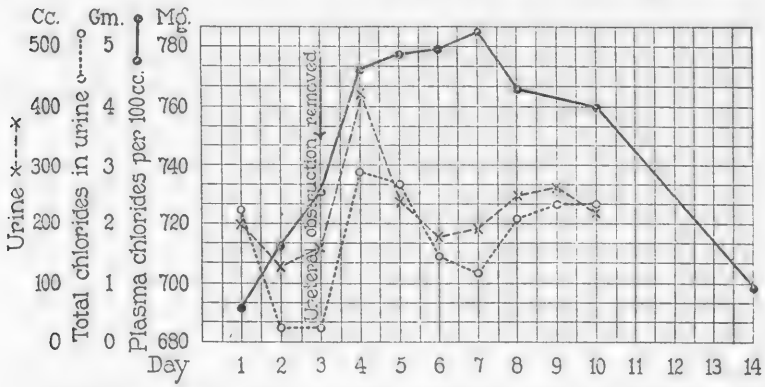


TEXT-FIG. 1. Dog 7. Plasma chlorides and blood urea in experimental hydronephrosis.

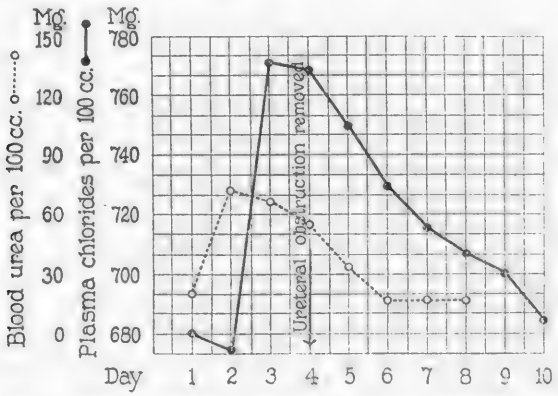
quickly reached the normal level, although the chloride content remained persistently elevated for several days. During this period the total renal function gradually improved and the chloride concentration in the urine soon rose to normal. Thus normal chloride excretion was restored before the plasma content had decreased to the preoperative level. McLean also observed in a case of acute nephritis the slow decrease in the plasma chlorides compared to the rapid decrease in blood urea.

In Dog 8, in which the renal impairment was less marked, both the plasma chlorides and urea increased rapidly before the ureteral obstruction was removed. This distinct, early increase in the plasma

chlorides (680 to 768 mg.) is in striking contrast to what occurred in the three previous experiments. On removing the ureteral bands, both the urea and chlorides rapidly diminished in the blood plasma and the normal urinary excretion of chlorides, urea, and phenolsul-



TEXT-FIG. 2. Dog 7. Plasma chlorides, volume of urine, and chlorides in the urine in experimental hydronephrosis.



TEXT-FIG. 3. Dog 8. Plasma chlorides and blood urea in experimental hydronephrosis.

fonephthalein was soon restored (Text-fig. 3). The ability of the kidneys to excrete an adequate amount of water during the entire experiment is in distinct contrast to the water retention that occurred in the first three experiments.

Observations on Animals with Chronic Hydronephrosis.

In a previous paper we demonstrated that animals with low grade ureteral obstruction gradually develop chronic hydronephrosis. Two such animals remained in good general health for from 4 to 5

TABLE IV.

Chronic Hydronephrosis.

Dog No.	Weight.	Date.	Plasma chlorides for each 100 cc.	Blood urea for each 100 cc.	Urine output.					
					Volume.	Specific gravity.	Sodium chloride.		Phenolsulfonephthalein excretion for 1 hr.	Urea for each 100 cc.
							Total amount.	Amount for each 100 cc.		
	kg.	*1916*	*mg.*	*mg.*	*cc.*		*gm.*	*gm.*	*per cent*	*gm.*
9	5.8	Jan. 23*	670	40	210	1.018	2.14	1.02	2	1.63
		" 24	680		105	1.023	1.18	1.12		0.81
		" 25	699	25	225	1.020	2.38	1.01		1.43
		" 26	690	25	265	1.020	2.65	1.00	28	0.99
	5.5	Feb. 19	685	46	220	1.027	0.79	0.36		2.33
		" 20	723		208	1.017	1.58	0.76		
		" 21	724	53	250	1.018	2.28	0.91	11	2.38
	6.0	Mar. 8	687		225	1.016	2.21	0.87		
		" 9	713		180	1.018	1.65	0.90		
		" 10	720	25	172	1.018	2.15	1.25		
	6.7	Apr. 5	629		156	1.019	1.69	1.09		
		" 6	627		225	1.022	2.77	1.23		
		" 7	654		170	1.018	2.03	1.20		
		" 8	685	30	220	1.022	2.14	0.97	33	
		" 20	635	25	190	1.020	2.40	1.26		2.01
		" 21	646		200	1.017	2.40	1.20		2.21
		" 22	626		197	1.017	2.44	1.23	28	

* 2 months since double ureteral obstruction was produced (operation November 20, 1915).

Intake 2 gm. of sodium chloride in 200 cc. of water daily, subcutaneously.

months, although during this entire period definite renal functional impairment could be demonstrated. These findings closely agree with the common clinical experience that patients with obstruction

in the lower urinary tract may have comparatively good health for months or years, even with marked pathologic changes in the kidneys and greatly reduced renal function. In one experiment (Dog 9, Table IV) studies in chloride metabolism were carried out on five occasions for periods of from 3 to 4 days. Food was not given, and sodium chloride and water were administered subcutaneously as outlined. In only two of these periods (February and March) were the total chloride excretion and concentration in the urine definitely reduced, and associated with this decreased chloride output the plasma chlorides showed a moderate rise. During the periods of study in January and April the plasma chloride content and the urinary output and concentration were within normal limits. Thus at the end of this experiment in April, in which renal function had been impaired for 5 months and marked anatomic changes had developed in the kidneys,[2] the chloride metabolism as evidenced by the concentration in the blood plasma and the total urinary output was normal. The concentration of the chlorides in the urine at this time tended to be somewhat low and uniform, but still above the minimal concentration noted in the series of normal controls under the same experimental conditions. It is an interesting fact that this dog excreted constantly a large volume of urine.

DISCUSSION.

The results obtained in these experiments emphasize the close relationship between the water and chloride equilibrium in the animal organism. With a constant moderate intake of water and sodium chloride, as administered in the control experiments, the chloride content of the plasma and the daily renal excretion of water and chlorides remain remarkably constant. This constancy of the chloride balance under normal conditions was the basis of the work of Ambard and Weill when they introduced their threshold formula for the excretion of chlorides. When this finely adjusted mechanism is disturbed by renal injury, as in these experiments by an acute or chronic hydronephrosis, the close interrelationship between the water

[2] See Keith, N. M., and Pulford, D. S., Jr., Experimental hydronephrosis. Functional and anatomic changes in the kidney following partial ureteral obstruction, *Arch. Int. Med.* 1917 xx, 864, Fig. 2.

and chloride balance is still evident, but certain distinctly abnormal changes are brought about. If water and chlorides are equally retained in the body, the plasma concentration of chlorides will not rise appreciably. This was the case in Dogs 5, 6, and 7 (Table III). Similar results were obtained by Leva in experimental uranium nephritis. The obvious reason for the rapid increase of the urea content of the tissues in spite of the retained water is that urea unlike the chlorides is being continually produced by the organism in large amounts. The interesting feature in three of the experiments is the striking rise in the plasma chloride content following the removal of the ureteral obstruction. This rise is chiefly due to the changes in renal function. The injured kidney has regained the ability to excrete water and urea readily, but chloride excretion, in contrast to water excretion, lags. The persistent retention of chlorides in the tissues is then indicated by a high plasma concentration, or threshold. The subsequent gradual fall in plasma chlorides to the normal level seems to be directly associated with the recovery of the specific renal function involved in chloride excretion. The rapid urea excretion after the removal of the ureteral obstruction closely follows the output of water. We should expect such a result because of the high urea content of the blood and the fact that urea in contrast to the chlorides is a non-threshold substance.

The experiment (Dog 8) furnishes a good example of simple or dry chloride retention. The kidney readily excretes water, but is unable to eliminate an adequate amount of chlorides. The resulting accumulation of chlorides in the body is indicated by an increased concentration in the plasma. Heineke and Meyerstein, Leva, and Austin and Jonas have shown that a similar rise in the blood or plasma chlorides takes place in experimental uranium nephritis when the intake of sodium chloride is large, as compared with that of water. Dry chloride retention has also been demonstrated in clinical cases of nephritis by Widal and Lemierre, Ambard and Beaujard, Leva, and others.

When the ureteral obstruction was removed in Dog 8 there was a rapid recovery of total renal function including the ability to excrete chlorides. The plasma chloride content fell more rapidly than in experiments on Dogs 5 and 7, but still did not reach the control level for several days after the blood urea was normal.

In the single experiment on chronic hydronephrosis (Dog 9) it is significant that the chloride metabolism under these experimental conditions deviated so little from the normal. The constant increased excretion of urine, or polyuria, such a common phenomenon in chronic renal lesions, appears to be the compensating mechanism which prevents any marked retention of chlorides, urea, or water. The renal cells in diffuse chronic nephritis work at a maximal excretory rate during the entire 24 hours, and thus for a time are able to rid the body of its accumulated excretory products. Probably, if more sodium chloride had been administered in the same volume of water during the foregoing experimental periods, a noticeable chloride retention would have resulted and caused a rise in the plasma chlorides.

In experiments on Dogs 6 and 7 the plasma chlorides do not show any appreciable increase during the initial period. The explanation for the failure of the plasma chlorides to rise when there was definite chloride retention would appear to be the dilution by the retained water of the tissue chlorides as a whole. This explanation might also account for the low plasma chloride concentration reported by Heineke and Meyerstein in experimental chromate and uranium nephritis and also in clinical cases of severe renal insufficiency. In the latter, low plasma chlorides have been noted by Lewis and Rivers in bichloride poisoning, by Major in chromic acid poisoning, and by Keith and Thomson in acute glomerular nephritis. In all these cases oliguria or anuria was present and the intake of fluids was high.

SUMMARY.

1. In acute experimental hydronephrosis chloride retention occurs as well as retention of water, urea, and phenolsulfonephthalein.

2. If both water and chlorides are retained there may be no appreciable rise in the plasma chloride content.

3. When chlorides are retained, but not water, the chloride content of the plasma rises strikingly.

4. After the removal of the ureteral obstruction in acute hydronephrosis all renal functions, water, urea, and chloride excretion, may be rapidly restored in equal degree, or the chlorides may be retained temporarily while there is free excretion of water and urea.

5. In chronic hydronephrosis adequate daily excretion of urea and chlorides may be maintained by a compensatory polyuria.

6. Chloride retention or an abnormal chloride excretion may occur in certain renal lesions when there is no change in the urea, phenolsulfonephthalein, or water excretion.

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BLOOD DESTRUCTION DURING EXERCISE.

III. Exercise as a Bone Marrow Stimulus.

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(Received for publication, August 25, 1922.)

Observations already recorded[1,2] offer strong evidence for the occurrence of blood destruction during exercise. There are yet other phenomena which point to the same conclusion. It is well known that any considerable loss of blood from hemorrhage or destruction of cells within the body is followed in the absence of complications by a definite increase in the number of reticulated red corpuscles in the peripheral circulation. This reaction can be taken therefore as a direct indication that unusual demands for new cells are being made upon the hematopoietic tissues. If exercise of caged animals produces such a well marked decrease in the red cell quantity as blood volume observations indicate,[2] a demand for corpuscles to replace this loss will inevitably result and this demand should be reflected in an increase in the number of reticulated cells.

Methods.

In order to rule out any possible influence upon the hematopoietic tissue of the methods employed to determine blood volume, a different series of animals was used in the present work from those heretofore reported upon.[1,2] Treadmill exercise was given them as already described. Dogs 17, 18, and 19 had been caged for only a little over a month at the time observations were begun. Nos. 20 and 21, by contrast, had been confined for over 4 months before the first observations and were caged and sedentary for a further 2 months prior to the second series. In enumerating the reticulated cells the method of Robertson[3] was employed. 10,000 cells were counted in each instance. The number of reticulocytes found in 10,000 red cells furnished the "count" of the animal. On

[1] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481.

[2] Broun, G. O., *J. Exp. Med.*, 1923, xxxvii, 113.

[3] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

the days on which exercise was given, at least two, and in some instances four, counts were made at intervals during the exercise period. The average of these counts has been employed for charting purposes. In the control periods before and after exercise only single counts were taken on any 1 day. Great individual differences were noted in the "normal" number of reticulocytes, but the variations occurring in the same animal from day to day, while considerable, were less marked.

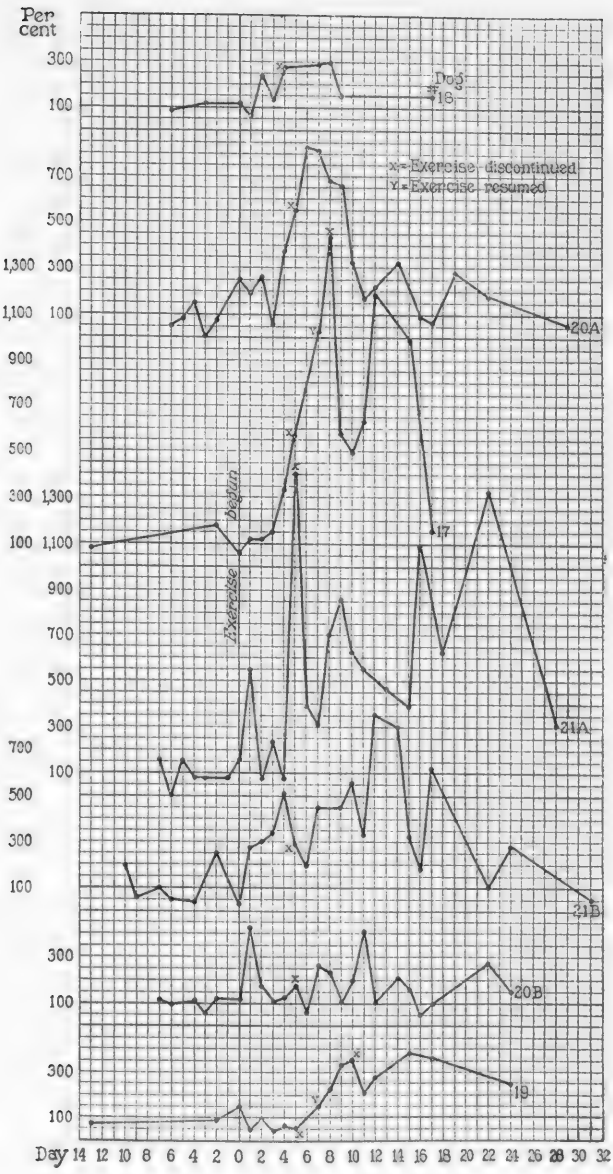
The reticulated elements were followed on all of the dogs for a week or more before exercise. The average of the counts was taken as the "normal" for the animal. In the tables, the counts are given as such, *i.e.* the number found in 10,000 red cells, as well as in percentages of the previous "normal." In the graphic representation in the charts, the variations are expressed in percentages of this "normal."

In the single experiment each on Nos. 17, 18, and 19, and in the last of the two experiments on Nos. 20 and 21, hemoglobin, hematocrit, and red cell determinations were made as well as the reticulated cell counts. Owing to the wide variations which result from changed cell distribution during a day of exercise, the blood was examined both before it and afterwards. The average of the two findings is used in the tables and charts. The hemoglobin percentage was determined by the method of Newcomer[4] in the case of Nos. 17, 18, and 19, and by the Palmer[5] method in the other two animals. Blood for the various counts was taken from the marginal ear vein of Nos. 20 and 21, and in the other animals from the external jugular.

Reticulated Cell Changes.

The reticulated cell counts are given in Table I and graphically portrayed in Text-fig. 1. No. 17 shows a well marked response to exercise, first manifest on the 5th day of it, and maintained throughout the following week. Thereafter only occasional observations were made; and when a practically normal count had been secured on the 10th day after exercise, they were discontinued. The animal had been confined but little over a month when exercise was begun. In No. 18, confined an equal length of time, the reaction, while distinct, assumes less considerable proportions. Only 5 days of exercise were given. No. 19, also caged for but a single month, showed no reaction during the 1st week of exercise. This was continued, therefore, into the 2nd week and a distinct increase in the reticulocyte count manifested itself. Dogs 20 and 21 had been caged over 4

[4] Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.
[5] Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.



TEXT-FIG. 1. Changes in reticulated red corpuscles during exercise.

TABLE I.

Alterations in the Number of Reticulated Red Corpuscles Resulting from Exercise.

Time.	No. 17. Male mongrel; weight 14¾ kilos.		No. 18. Male mongrel; weight 11¾ kilos.		No. 19. Male mongrel; weight 14½ kilos.		No. 20 (Experiment A). Female mongrel; weight 16 kilos.		No. 20 (Experiment B). Female mongrel; weight 16 kilos.		No. 21 (Experiment A). Female mongrel; weight 7 kilos.		No. 21 (Experiment B). Female mongrel; weight 7 kilos.		Daily average count of series.	Daily average of per cent of normal average figures.	Remarks.
	Count.	Per cent of normal average.	Count.	Per cent of normal average.	Count.	Per cent of normal average.	Count.	Per cent of normal average.	Count.	Per cent of normal average.	Count.	Per cent of normal average.	Count.	Per cent of normal average.			
13 days before beginning exercise...	7	72			12	72									9	72	No exercise.
10 " " " " ..													16	190	16	190	" "
9 " " " " ..													5	59	5	59	" "
7 " " " " ..									12	111	2	156	8	95	7	121	" "
6 " " " " ..			11	83			5	52	10	92	0	0	4	47	6	55	" "
5 " " " " ..							8	83			2	156			5	119	" "
4 " " " " ..							14	145	11	102	1	78	3	36	7	90	" "
3 " " " " ..			14	105			0	0	6	55	1	78			5	60	" "
2 " " " " ..	17	176			14	84	7	72	13	120			21	249	14	140	" "
1 day " " " ..											1	78			1	78	" "
The day exercise was begun.....	5	52	15	113	24	144	24	248	13	120	2	156	2	24	12	122	All animals exercised 4 hrs.
1 day later.....	11	114	8	60	7	42	18	186	46	425	7	545	23	273	17	235	All animals exercised 4 hrs.
2 days "	11	114	31	233	16	96	25	258	19	175	1	78	25	296	18	179	All animals exercised 4 hrs.
3 " "	14	145	18	135	7	42	5	52	12	111	3	233	28	332	12	150	All animals exercised 4 hrs.

4 days later			32	331	36	270	11	66	36	372	14	129	1	78	43	510	25	251	All animals exercised 4 hrs.
5	"	"	54	559			9	54	53	548	19	175	18	1,400	24	285	29	504	No. 18 not exercised. All others exercised 2 hrs.
6	"	"							80	828	7	65	5	389	16	190	27	366	Animals not exercised.
7	"	"	99	1,024	37	278	26	156	79	817	28	258	4	311	38	451	44	471	Nos. 17 and 19 exercised 4 hrs. Others not exercised.
8	"	"	138	1,428	39	293	38	228	65	672	25	231	9	700			52	592	No. 19 exercised 4 hrs. Others not exercised.
9	"	"	55	569	20	150	54	324	63	652	12	111	11	857	38	451	36	445	No. 19 exercised 4 hrs. Others not exercised.
10	"	"	48	497			58	348	31	320	21	194	8	622	47	558	36	423	No. 19 exercised 4 hrs. Others not exercised.
11	"	"	61	631			35	210	16	165	44	406	7	545	28	332	32	382	Exercise discontinued on all animals.
12	"	"	114	1,180			46	276	21	216	11	102			72	854	53	525	No exercise.
13	"	"											6	467			6	467	" "
14	"	"							31	321	22	203			67	794	40	439	" "
15	"	"	95	983			63	378			17	157	5	389	27	322	41	446	" "
16	"	"							8	83	5	46	14	1,090	15	178	10	349	" "
17	"	"	15	155	20	150	60	360	6	62	10	93			52	617	27	239	" "
18	"	"											8	622			8	622	" "
19	"	"							27	279							27	279	" "
22	"	"							17	176	29	268	17	1,323	9	107	18	469	" "
24	"	"					40	240			16	148			23	273	26	220	" "
28	"	"											4	311			4	311	" "
29	"	"							5	52							5	52	" "
31	"	"													4	47	4	47	" "

The reticulated cell count as given represents the number of reticulated corpuscles in 10,000 red corpuscles.

months at the time of the first experiment upon them. The reticulocyte curve of No. 20 shows a well marked reaction to exercise at this time. On exercise again 2 months later, a definite reaction was secured but one much less imposing than had previously occurred. Prior to exercise the blood of No. 21 showed but a very small number of reticulocytes, 2 in 10,000 being the greatest number noted. Though the actual counts after exercise were not high, they amount in percentage terms to a very large increase. This increase was maintained practically throughout the 2 months elapsing before the second experiment. Nevertheless, during the second exercise period there was a marked reticulocyte rise above the already heightened level.

Several interesting points deserve mention in connection with the character of the reaction. First of all, the irregular wave-like character of curves derived from successive reticulated cell counts under ordinary circumstances should be noted. A single count is no reliable index to this curve. In some instances (Text-fig. 1, Nos. 21A and 20B) a slight increase in the reticulocyte count occurred during the 1st day or two of exercise. This was followed by a distinct fall in the count and only towards the end of the exercise period did a second rise occur. In practically every instance the crest of the rise is reached after exercise has been discontinued, and the curve remains relatively high until hemoglobin and cell count have come back within normal limits. It may well be that the rise noted early in the course of exercise represents the appearance of a certain reserve supply of cells held in the bone marrow for emergency purposes and that these disappear from circulation during the following days, when the activity of the marrow has not yet increased. With this increase there occurs a second rise, near the end of the 1st week of exercise.

Changes Shown by the Hemoglobinometer, Hematocrit, and Red Count.

In Tables II to IV and in Text-fig. 2 are given the data obtained by the use of hemoglobinometer, hematocrit, and red counts on the series of animals already dealt with. It will be noted that the findings are in complete general agreement with the results of blood volume determinations as previously reported upon, in that they indicate the disappearance of a considerable quantity of blood during exercise.[2]

Taken independently the figures would not offer conclusive proof of this destruction as they do not deal with total values for the body. Certain irregular fluctuations that will be noted may be due either to errors in the individual determinations or to local or general changes in the ratio of fluid to cells. For example, a persistently high plasma volume will suffice to account for the fact that the depression of these curves persisted for nearly 3 weeks after exercise had ceased. In Text-fig. 3 is given a composite picture of the changes in reticulocytes, hemoglobin, hematocrit, and red count. Whenever counts were made on more than one dog on the same day the percentage figures yielded by these counts were averaged and used for the composite chart. The general features of the curves leave room for no doubt of the changes.

It is *a priori* possible that the reticulated cell reaction may be due to other causes than the stimulus of blood loss, as for example, to some chemical substance engendered or liberated during exercise. If it be due on the other hand wholly to cell loss, then on prompt replacement of this loss from outside sources the rise in reticulocytes should not occur. To test the point the following experiment was performed.

Preliminary blood volume determinations were made on two dogs previously caged during 2 months. Hemoglobin and reticulated cell counts were taken for 2 days beforehand as well as daily during the course of exercise. The animals were exercised on treadmills after the usual manner for 5 days. The reticulated cell count showed at the end of this time an upward trend in both animals. A second blood volume determination was now carried out and the quantity of cells lost determined. Each animal was now transfused with blood from a compatible donor to the amount that should on calculation exactly replace the loss of hemoglobin. No further exercise was given.

The results of this experiment are presented in Table V and Text-fig. 4. It will be seen that the usual increase in reticulocytes after exercise did not occur. In fact the cell count fell below the previous level.

Yet another test:—If the increase in reticulated cells is due to anemia from blood destruction, an animal made plethoric artificially should on exercise show no rise in reticulocytes so long as the blood amount remains abnormally large.

TABLE II.

Changes in Hemoglobin Percentage Resulting from Exercise.

Time.	No. 17. Male mongrel; weight 14¾ kilos.		No. 18. Male mongrel; weight 11½ kilos.		No. 19. Male mongrel; weight 14½ kilos.		No. 20 (Experiment B). Female mongrel; weight 16 kilos.		No. 21 (Experiment B). Female mongrel; weight 7 kilos.		Daily average hemoglobin per cent of series.	Daily average of per cent of normal average figures.	Remarks.
	Per cent hemoglobin.	Per cent of normal average.	Per cent hemoglobin.	Per cent of normal average.	Per cent hemoglobin.	Per cent of normal average.	Per cent hemoglobin.	Per cent of normal average.	Per cent hemoglobin.	Per cent of normal average.			
13 days before beginning exercise. .	133	99			137	100					135	99	No exercise.
10 " " " " . .									109	98	109	98	" "
9 " " " " . .									109	98	109	98	" "
7 " " " " . .							107	98	107	96	107	97	" "
6 " " " " . .			124	98			114	104	111	100	116	101	" "
5 " " " " . .							111	102			111	102	" "
4 " " " " . .							106	97	108	97	107	97	" "
3 " " " " . .			130	102			109	100			119	101	" "
2 " " " " . .	134	100			140	102	107	98	116	104	124	101	" "
The day exercise was begun.....	136	101	127	100	135	98	111	102	119	107	126	102	All animals exercised 4 hrs.
1 day later.....	117	87	114	90	125	91	108	99	109	98	115	93	" " " 4 "
2 days "	117	87	119	94	129	94	107	98	108	97	116	94	" " " 4 "
3 " "	118	88	105	83	128	93	106	97	112	101	114	92	" " " 4 "
4 " "	111	83	115	91	120	88	100	92	106	95	110	90	" " " 4 "

5 days later.....	108	80			124	90	98	90	106	95	109	89	No. 18 not exercised. All others exercised 2 hrs.
6 " "													Animals not exercised.
7 " "	102	76	115	91	130	95	97	89	101	91	109	88	Nos. 17 and 19 exercised 4 hrs. Others not exercised.
8 " "	108	80	117	92	114	83	92	84			108	85	No. 19 exercised 4 hrs. Others not exercised.
9 " "	120	89	118	93	123	90	97	89	99	89	111	90	No. 19 exercised 4 hrs. Others not exercised.
10 " "	119	89			125	91	87	80	102	92	108	88	No. 19 exercised 4 hrs. Others not exercised.
11 " "	116	86			120	88					118	87	Exercise discontinued on all animals.
12 " "	113	84			120	88	90	82	104	94	107	87	No exercise.
15 " "	115	86			133	97	94	86	105	94	112	91	" "
17 " "	123	92	129	101	133	97	96	88	105	94	117	94	" "
22 " "	118	88					97	89	104	94	106	90	" "
24 " "					126	92	94	86	117	105	112	94	" "
31 " "									114	102	114	102	" "

The per cent of hemoglobin is given in terms of the Haldane scale.

TABLE III.

Changes in the Plasma-Cell Ratio Resulting from Exercise.

Time.	No. 17. Male mongrel; weight 14¼ kilos.		No. 18. Male mongrel; weight 11¾ kilos.		No. 19. Male mongrel; weight 14½ kilos.		No. 20 (Experiment B). Female mongrel; weight 16 kilos.		No. 21 (Experiment B). Female mongrel; weight 7 kilos.		Daily average volume per cent of cells for the series.	Daily average of per cent of normal average figures.	Remarks.
	Volume per cent of cells.	Per cent of normal average.	Volume per cent of cells.	Per cent of normal average.	Volume per cent of cells.	Per cent of normal average.	Volume per cent of cells.	Per cent of normal average.	Volume per cent of cells.	Per cent of normal average.			
5 days before beginning exercise. . .							48.2	97			48.2	97	No exercise.
4 " " " " . . .							51.0	102			51.0	102	" "
3 " " " " . . .			48.3	99			49.0	98			48.6	98	" "
2 " " " " . . .	51.2	99							54.0	101	52.6	100	" "
The day exercise was begun.	52.0	101	49.5	101	50.9	100	51.0	102	52.5	99	51.2	101	All animals exercised 4 hrs.
1 day later.	44.2	86	45.0	92	45.4	89	48.2	97	47.8	90	46.1	91	" " " 4 "
2 days "	43.9	85	45.8	94	45.7	90	45.8	92	49.3	93	46.1	91	" " " 4 "
3 " "	41.6	81	44.2	90	43.6	86	44.4	89	48.4	91	44.4	87	" " " 4 "
4 " "	43.9	85	42.9	88	48.6	96	43.8	88	45.2	85	44.9	88	" " " 4 "
5 " "	44.5	86			49.7	98	40.5	81	44.3	83	44.7	87	No. 18 not exercised. All others exercised 2 hrs.
6 " "													Animals not exercised.
7 " "	46.1	89	44.7	91	50.7	100	39.9	80	44.8	84	45.2	89	Nos. 17 and 18 exercised 4 hrs. Others not exercised.
8 " "	42.0	81	43.6	89	48.6	96	40.7	82			43.7	87	No. 19 exercised 4 hrs. Others not exercised.

9 days later.....	42.2	82	44.3	91	48.1	95	39.4	79	40.8	77	43.0	85	No. 19 exercised 4 hrs. Others not exercised.
10 " "	43.7	85			48.0	94	41.8	84	47.7	90	45.3	88	No. 19 exercised 4 hrs. Others not exercised.
11 " "							40.5	81	43.5	82	42.0	81	Exercise discontinued on all animals.
12 " "	43.8	85			50.9	100	43.8	88	46.9	88	46.3	90	No exercise.
15 " "	44.4	86			52.4	103	41.0	82	48.4	91	46.5	90	" "
17 " "	44.9	87	46.6	95	51.2	101	42.1	85	50.7	95	47.1	93	" "
22 " "	44.3	86					43.6	88	47.9	90	45.3	88	" "
24 " "					49.3	97	43.8	88	53.5	100	48.9	95	" "
31 " "									54.6	103	54.6	103	" "

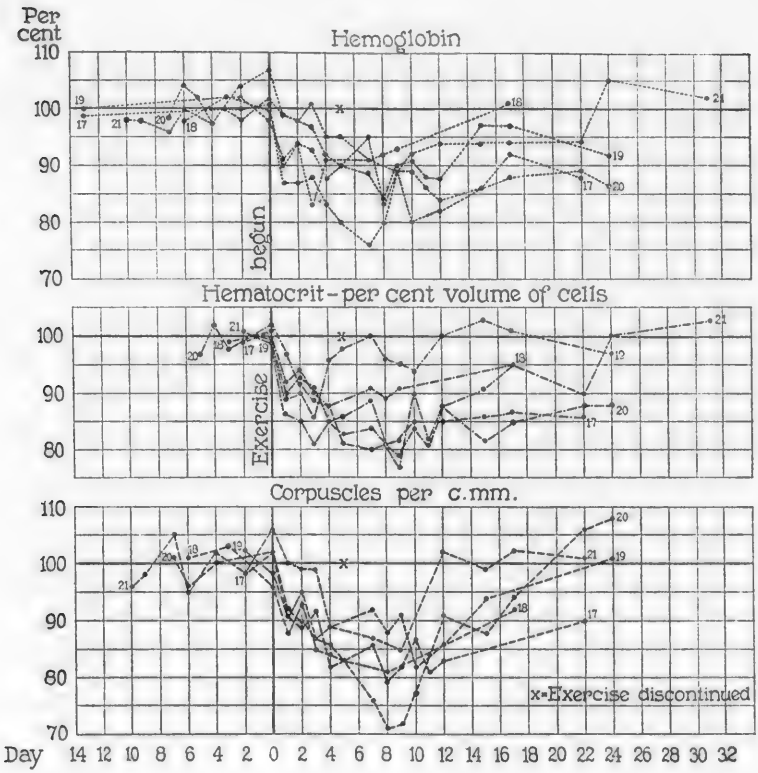
The hematocrit determinations were made by the method of Epstein.

TABLE IV.

Changes in the Number of Corpuscles per Cubic Millimeter Resulting from Exercise.

Time.	No. 17. Male mongrel; weight 14¾ kilos.		No. 18. Male mongrel; weight 11¾ kilos.		No. 19. Male mongrel; weight 14½ kilos.		No. 20 (Experiment B). Female mongrel; weight 16 kilos.		No. 21 (Experiment B). Female mongrel; weight 7 kilos.		Daily average red count of series.	Daily average of per cent of normal average figures.	Remarks.
	Red count.	Per cent of normal average.	Red count.	Per cent of normal average.	Red count.	Per cent of normal average.	Red count.	Per cent of normal average.	Red count.	Per cent of normal average.			
10 days before beginning exercise...									8,030,000	96	8,030,000	96	No exercise.
9 " " " " ...									8,220,000	98	8,220,000	98	" "
7 " " " " ...							8,520,000	101	8,780,000	105	8,650,000	103	" "
6 " " " " ...			8,930,000	101			8,100,000	96	8,000,000	95	8,340,000	97	" "
4 " " " " ...							8,460,000	100	8,550,000	102	8,500,000	101	" "
3 " " " " ...			9,120,000	103							9,120,000	103	" "
2 " " " " ...	8,980,000	98			9,670,000	102			8,200,000	98	8,950,000	99	" "
The day exercise was begun.....	9,320,000	102	8,510,000	96	9,230,000	98	8,600,000	102	8,870,000	106	8,910,000	101	All animals exercised 4 hrs.
1 day later.....	8,430,000	92	7,830,000	88	8,560,000	91	7,680,000	91	8,420,000	100	8,180,000	92	All animals exercised 4 hrs.
2 days "	8,220,000	90	8,220,000	93	9,000,000	95	7,510,000	89	8,320,000	99	8,250,000	93	All animals exercised 4 hrs.
3 " "	7,930,000	87	7,550,000	85	8,200,000	87	7,730,000	92	8,300,000	99	7,940,000	90	All animals exercised 4 hrs.

4 days later	7,870,000	86	7,450,000	84	8,400,000	89	6,880,000	82	7,500,000	89	7,620,000	86	All animals exercised 4 hrs.
5 " "	7,630,000	83					7,030,000	83			7,330,000	83	No. 18 not exercised. All others exercised 2 hrs.
6 " "													Animals not exercised.
7 " "	7,830,000	86			8,720,000	92	6,390,000	76	7,300,000	87	7,560,000	85	Nos. 17 and 19 exercised 4 hrs. Others not exercised.
8 " "	7,260,000	79	7,190,000	81	8,330,000	88	5,980,000	71			7,190,000	80	No. 19 exercised 4 hrs. Others not exercised.
9 " "	7,550,000	83			8,610,000	91	6,100,000	72	7,100,000	85	7,340,000	83	No. 19 exercised 4 hrs. Others not exercised.
10 " "	7,960,000	87			7,770,000	82	6,450,000	77			7,390,000	82	No. 19 exercised 4 hrs. Others not exercised.
11 " "	7,430,000	81			7,890,000	84					7,660,000	82	Exercise discontinued on all animals.
12 " "	7,580,000	83			8,160,000	86	7,650,000	91	8,590,000	102	8,000,000	90	Exercise discontinued on all animals.
15 " "					8,890,000	94	7,440,000	88	8,260,000	99	8,200,000	94	Exercise discontinued on all animals.
17 " "			8,180,000	92			7,890,000	94	8,570,000	102	8,210,000	96	Exercise discontinued on all animals.
22 " "	8,200,000	90					8,950,000	106	8,500,000	101	8,550,000	99	Exercise discontinued on all animals.
24 " "					9,600,000	101	9,150,000	109			9,370,000	105	Exercise discontinued on all animals.



TEXT-FIG. 2. Changes during exercise shown by the hemoglobinometer, hematocrit, and red count.

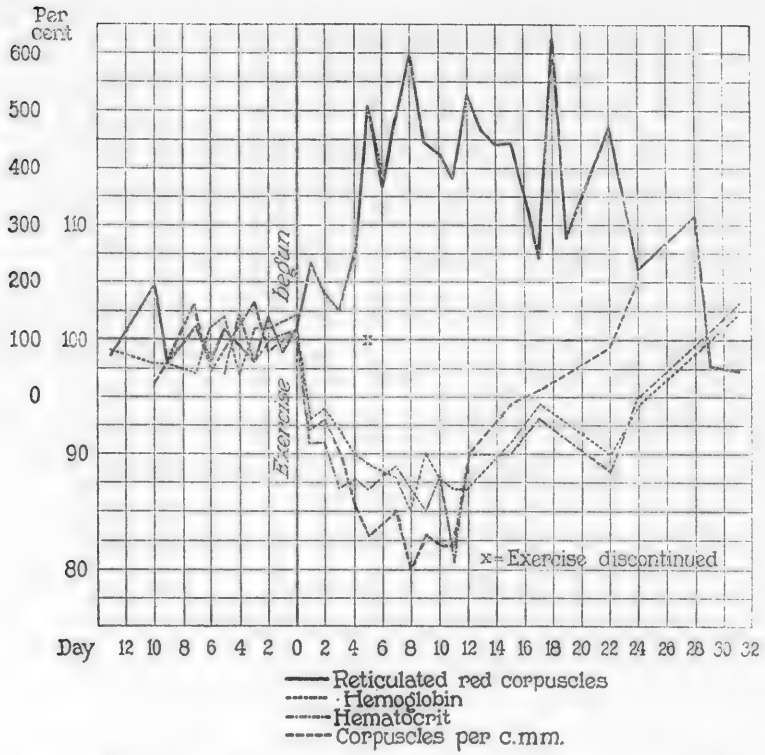
In Dog 26, a normal animal caged for 3 months, the number of reticulocytes, per cent of hemoglobin, and number of red cells per cubic millimeter were followed for 2 weeks. The animal was then given intravenously 550 cc. of citrated blood from a compatible donor. This caused definite plethora. A period of 48 hours was allowed for readjustment and then exercise was begun and kept up for 6 days. The blood volume was not followed.

As the chart shows (Table VI, Text-fig. 5), a slight fall in red count and hemoglobin occurred during the exercise period, but both remained above the normal level. The reticulated cells disappeared

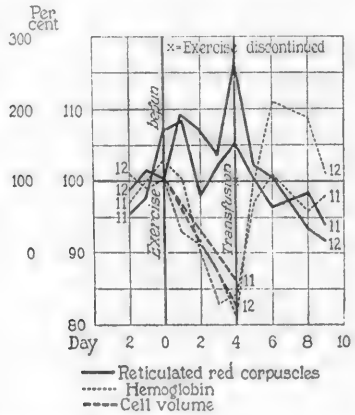
TABLE V.

Prevention of the Reticulated Cell Reaction by Transfusion.

Time.	No. 11. Male setter; weight 19½ kilos.								No. 12. Male mongrel; weight 16½ kilos.								Remarks.
	Reticulated red corpuscles.		Hemoglobin.		Cell volume.		Plasma volume.		Reticulated red corpuscles.		Hemoglobin.		Cell volume.		Plasma volume.		
	No. in 10,000 red cells.	Per cent of normal average.	Per cent.	Per cent of normal average.	Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	No. in 10,000 red cells.	Per cent of normal average.	Per cent.	Per cent of normal average.	Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
2 days before beginning exercise..	26	55	125	97					19	86	129	101					No exercise.
1 day " " " .	36	76							25	114	126	99					" "
The day exercise was begun.....	80	169	134	103	863	100	918	100	22	100	127	100	760	100	848	100	Both animals exercised 4 hrs. after blood volume determination.
1 day later.....	86	182	129	100					42	191	118	93					Both animals exercised 4 hrs. after blood volume determination.
2 days "	39	83	116	90					38	173	116	91					Both animals exercised 4 hrs. after blood volume determination.
3 " "	59	125	107	83					30	136	110	86					Both animals exercised 4 hrs. after blood volume determination.
4 " "	73	154	108	84	740	86	1,052	115	58	264	103	81	624	82	948	112	Both animals exercised 2 hrs. Blood volume determination made at end of this period and each animal was transfused. No. 11 received 284 cc. of blood of hemoglobin value of 120 per cent. No. 12 received 309 cc. of the same blood. Exercise discontinued after transfusion.
5 " "	49	104	125	97					26	118	127	100					
6 " "	31	66	130	100					23	105	141	111					
8 " "	40	85	124	96					8	36	137	108					
9 " "	19	40	126	97					4	18	130	102					



TEXT-FIG. 3. General comparison of the changes in the reticulated red corpuscles with the hemoglobin, red count, and hematocrit.



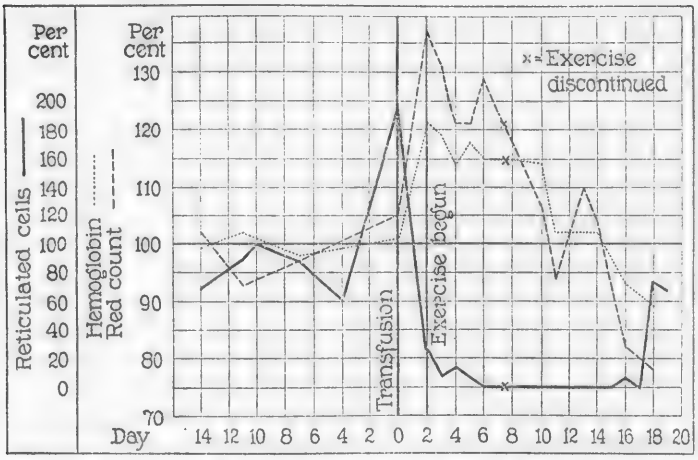
TEXT-FIG. 4. Prevention of the reticulated cell reaction by transfusion.

TABLE VI.

Absence of the Reticulocyte Reaction to Exercise in a Plethoric Animal.

Time.	No. 26. Male setter; weight 18½ kilos.						Remarks.
	Reticulated red corpuscles.		Hemoglobin.		Red count.		
	No. in 10,000 red cells.	Per cent of pretransfusion average.	Per cent.	Per cent of pretransfusion average.	Corpuscles per c. mm.	Per cent of pretransfusion average.	
14 days before transfusion.....	10	68	101	99	9,100,000	102	No exercise.
11 " " "	13	88	104	102	8,300,000	93	" "
10 " " "	15	101					" "
7 " " "	13	88	100	98			" "
4 " " "	9	61					" "
The day of transfusion.....	29	196	103	101	9,370,000	105	" " Transfused 550 cc. of blood.
2 days later.....	4	27	123	121	12,210,000	137	Exercised 4 hrs.
3 " "	1	7	121	119	11,700,000	131	" 4 "
4 " "	2	14	116	114	10,800,000	121	" 4 "
5 " "	1	7	120	118	10,800,000	121	" 4 "
6 " "	0	0	117	115	11,500,000	129	" 4 "
7 " "	0	0	117	115	11,000,000	123	" 3 "
9 " "	0	0					No exercise.
10 " "	0	0	116	114	9,500,000	106	" "
11 " "	0	0	104	102	8,400,000	94	" "
13 " "	0	0	104	102	9,800,000	110	" "
14 " "	0	0	104	102	9,300,000	104	" "
15 " "	0	0					" "
16 " "	1	7	95	93	7,380,000	83	" "
17 " "	0	0					" "
18 " "	11	74	91	89	6,990,000	78	" "
19 " "	10	68					" "

practically completely from the blood stream within 3 days after the transfusion and did not reappear during the exercise nor for more than a week thereafter. By this time the red counts and hemoglobin determinations had fallen below the normal level and in the course of the next few days reticulated cells again appeared.



TEXT-FIG. 5. Absence of reticulocyte rise after exercise in a plethoric animal.

DISCUSSION.

The foregoing observations, in connection with those previously reported, yield a number of suggestions as to the reaction of the bone marrow of caged animals to exercise. Obviously there is at first a period in which blood destruction is proceeding more rapidly than blood formation. In most animals during this period the reticulated count is increased but little. The need for new cells is then as great or greater than during the following week when the animal is again resting. It is possible that reticulated cells are broken up more quickly during exercise than normal cells. However, in this case the counts prior to each day of exercise should be higher than those at the end of it, and this was not regularly the case. One may believe that during the long period of confinement which preceded exercise the bone marrow had become inert, perhaps even atrophied, as com-

pared with that of normal animals and it was for a time unable to produce new cells to meet the sudden demand. More will be said upon the point in a paper that is to follow.[6]

Unfortunately no reticulated cell observations were made in which the period of exercise was extended over several weeks. It is probable that as the marrow adapts itself to the demands upon it, the number of reticulocytes will fall to the normal.

The observations of this and the preceding papers taken together strongly indicate that the rôle of exercise as a pace maker in blood destruction and formation is an important one. Just as a disused muscle becomes weakened, and atrophied, so presumably may a bone marrow which is not constantly working to replace worn out cells lessen also in its functional ability. And with the sudden increase in blood destruction brought about by exercise there ensues what may be termed marrow decompensation.

As everyone knows, exercise is good for the body in many ways. One important effect would seem to be a stimulation of the hematopoietic system to a high efficiency. This stimulus need not take the form of a reduction in the gross number of red cells through injury to them, unless indeed the exercise is so overdone that blood destruction exceeds the reparative abilities of the marrow. The loss of blood that has been demonstrated in sedentary animals[2] produces a temporary anemia in them only because their hematopoietic tissues are not in a condition to compensate for the increased corpuscular wear and tear. With mild exercise the increases in cell destruction and production should take place *pari passu*. And the result will be a marrow fit for emergencies.

There are, of course, other stimuli to the hematopoietic tissues from exercise besides the loss of blood cells. One thinks immediately of the relative oxygen lack consequent on increased metabolism. Air-hunger during violent exercise is often as great as that at high altitudes, and the resulting marrow stimulation should be as considerable. But this and kindred matters need not enter into present consideration.

The way in which the red cells are destroyed in increased quantity during exercise remains undetermined. Rous and Robertson[7] have

[6] Broun, G. O., *J. Exp. Med.*, 1923, xxxvii, 207.

[7] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

brought forward evidence for the view that normal blood destruction, and that of some pathological states as well, occur through a mechanical fragmentation of the erythrocytes. It was on the assumption that destruction of this nature would be increased by the wear and tear incident to the quickened blood flow of exercise that the present work was undertaken. The assumption has been borne out in that an increased destruction has been found. But whether the latter comes about by corpuscular fragmentation remains to be seen.

SUMMARY.

A definite increase in the percentage of reticulocytes occurs after exercise in animals previously kept to a sedentary life. Concomitant changes in the hemoglobin percentage, plasma-cell ratio, and red count offer evidence in addition to that already reported by the author of the occurrence of an increase in blood destruction under such circumstances.

Replacement by transfusion of blood destroyed during exercise prevents the reticulated cell reaction. Animals rendered plethoric and then exercised show no increase in reticulated cells while the plethora persists.

The fact is emphasized that exercise must be an important factor in the maintenance of an efficient hematopoietic tissue.

BLOOD DESTRUCTION DURING EXERCISE.

IV. The Development of Equilibrium between Blood Destruction and Regeneration after a Period of Training.

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(Received for publication, August 25, 1922.)

It is obvious that in normally active individuals a practically constant balance must be maintained between blood destruction and blood formation in spite of occasional unusual exertions such as may be supposed to cause a temporary increase in blood destruction.[1,2,3] Experiments described in previous communications[1,2,3] show that this is not the case when animals which have been caged for some months are suddenly subjected to strenuous exercise during several days. In them there appears a condition which may best be described as marrow decompensation. Blood is destroyed more rapidly than the hematopoietic tissue can replace it and a well marked fall in cell volume and total circulating hemoglobin is the result. The marrow gives evidence that increased demands are being made upon it by placing increased numbers of reticulated red corpuscles in circulation. If exercise is discontinued after 5 or 6 days, the cell volume fails to return to its initial level until from 1 to 3 weeks have elapsed.

The exercise to which the animals were subjected was fairly severe. Would the changes have occurred had the dogs been trained as athletes before entering upon so strenuous a test? The present observations will deal first with the reaction to exercise of animals confined for but a brief period and therefore moderately fit; and, second, with the changes in dogs put through a course of "training" of several weeks duration.

[1] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481.
[2] Broun, G. O., *J. Exp. Med.*, 1923, xxxvii, 113.
[3] Broun, G. O., *J. Exp. Med.*, 1923, xxxvii, 187.

Experimental Procedures.

The methods used for estimating blood volume, the general care of the animals, and the routine procedure of exercise have already been described.[1,2]

The dogs of Series A (Table I and Text-fig. 1) were exercised 2 weeks after their arrival in the animal house. It was not possible to ascertain how long or how closely they had been confined prior to this time. However, they undoubtedly approximated normally active animals more closely than did the dogs employed in the previous experiments,[2] since these had all been caged for several months. Exercise on treadmills was given in the usual manner for 5 days. The blood volume was followed by the dye method.

The animals of Series B (Table II and Text-fig. 2) had been utilized for experiments described in a previous paper.[4] As it is important to show the relation of these experiments to the later ones, some of the data from the former are included in the present table and chart. The dogs were allowed to recover from the first period of exercise, and, after an interval of caging, again run on the treadmill during 1 to 5 weeks. In the first period of exercise volume determinations were made by the CO method only. In the subsequent exercise periods both methods were employed on all animals save No. 2. The results agree closely.

Effect of Exercise after Brief Confinement.

The findings in the animals exercised after brief confinement are recorded in Table I and graphically represented in Text-fig. 1. It will be seen that after 5 days of exercise a slight decrease in cell volume occurred in each instance. Dogs 13 and 14 showed a well marked fall in cell volume on the 4th and 3rd days of exercise respectively. On the 5th day both had to a great extent recovered. No. 15 exhibited a slight increase in cell volume on the 3rd day of exercise but by the 5th day this had fallen 10 per cent below the normal. No. 16 showed a decrease of 11 per cent on the last day of exercise. The average decrease for the series after the 5 days of exercise amounts to 11 per cent. Animals previously caged for several months show under similar circumstances an average decrease of 19 per cent.[2] A distinct difference in the magnitude of the change in cell volume is therefore to be noted. The observations on the present series of animals were not continued beyond the exercise period.

[4] Broun,[2] Series A.

Prolonged Exercise in Sedentary Animals.

The results of the experiments on prolonged exercise are given in Table II and Text-fig. 2. The data on each animal will be considered separately.

Dog 0 had been caged 5 months when observations upon it were begun. During the first period of exercise, in which the blood volume determinations were made by the CO method, the cell volume fell 15 per cent below the normal for the animal. 19 days later it had returned to within 3 per cent of the normal and in the following 5 days had risen to 2 per cent above. At this time the animal was again exercised for 4 days. No decrease in cell volume occurred. On the contrary a rise to 107 per cent was found at the end of the period. The next day two volume determinations were made, one by the CO method which yielded volume equivalent to 109 per cent of the normal and one by the dye method which gave a value of 101 per cent. The animal was then caged for 39 days, then exercised 4 hours daily on 6 days of each week for 3 weeks. Determinations at the end of this time, both by the CO and the dye methods, showed a cell volume 11 per cent higher than the normal established when observations were begun.

Animal 1 was caged 3 months before the first exercise period. 6 days exercise at this time caused a 12 per cent decrease in cell volume. Within 6 days thereafter the cell volume rose to 1,425 cc., or 115 per cent of the normal, as determined by the CO method, a remarkably high figure for a 22 kilo animal. A dye volume determination on the same day gave a cell volume of 1,450 cc. and on the following day another CO determination showed a cell volume of 1,428 cc. The three determinations agree so closely that experimental error can be eliminated as an explanation for the large volume found. Next day exercise was begun and the animal was run on the treadmill 4 hours daily for 4 days in that week and for 6 days in each of the 4 following weeks. At the end of the 2nd week of exercise a determination by the dye method showed that the cell volume had fallen from the high level of 117 per cent to exactly the normal average. No CO determination was made at this time. At the end of the 3rd week a dye volume determination yielded a cell volume equivalent to

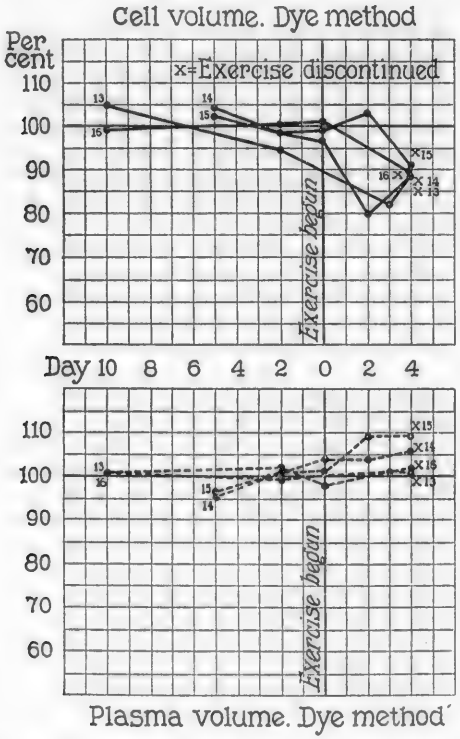
TABLE I.

Series A. Effect of Exercise after Brief Confinement.

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
						Animal had been caged 2 wks. at the time observations were begun.
No. 13. Male mongrel; weight 12¾ kilos.	10 days before beginning exercise.	626	105	862	101	10 min. exercise before blood volume determination. Otherwise no exercise.
	2 " " " "	567	95	853	99	10 min. exercise before blood volume determination. Otherwise no exercise. 2 days later exercise was begun and animal was exercised 4 hrs. daily for 5 days.
	After 4 days of exercise.	491	82	865	101	Blood volume determination at end of exercise period.
	" 5 " " "	525	88	864	101	Blood volume determination at end of exercise period.
						Animal had been caged 2 wks. at the time observations were begun.
No. 14. Male mongrel; weight 13¾ kilos.	5 days before beginning exercise.	671	104	799	96	10 min. exercise before blood volume determination. Otherwise no exercise.
	2 " " " "	633	98	845	101	10 min. exercise before blood volume determination. Otherwise no exercise.
	The day exercise was begun.	624	97	862	103	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.

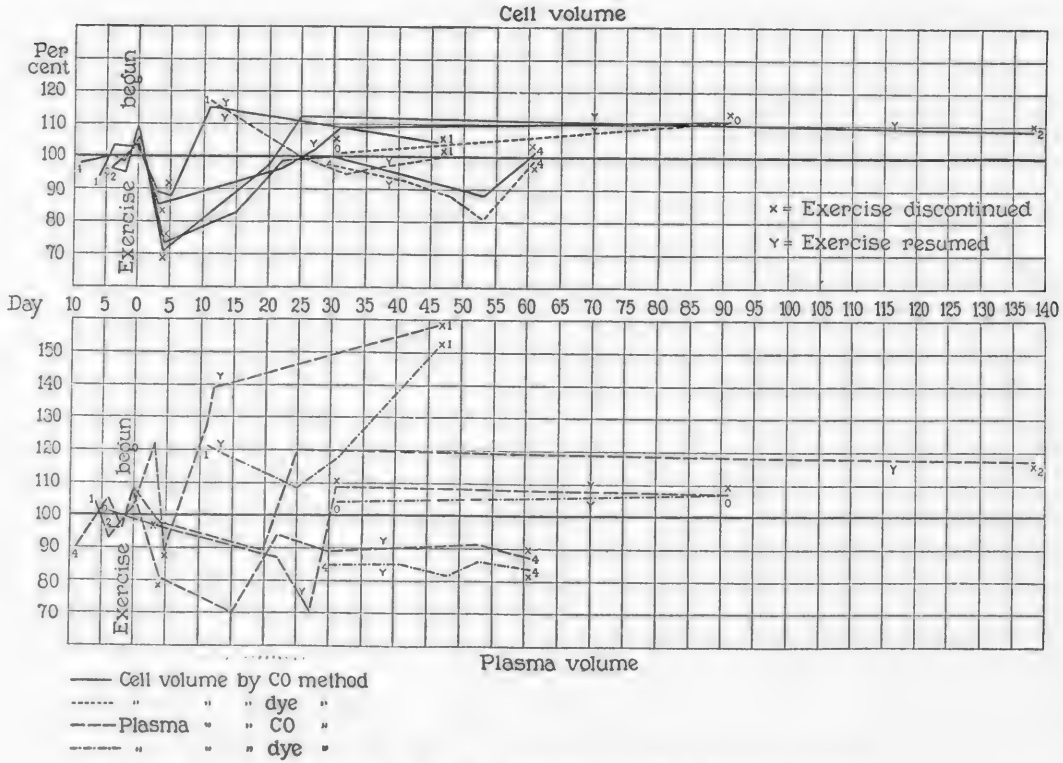
	After 3 days of exercise.	515	80	865	104	Blood volume determination at end of exercise period.
	" 5 " " "	570	89	888	107	Blood volume determination at end of exercise period.
						Animal had been caged 2 wks. at the time observations were begun.
No. 15. Male mongrel; weight $12\frac{3}{4}$ kilos.	5 days before beginning exercise.	694	103	626	96	10 min. exercise before blood volume determination. Otherwise no exercise.
	2 " " " "	657	98	660	102	10 min. exercise before blood volume determination. Otherwise no exercise.
	The day exercise was begun.	660	99	660	102	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.
	After 3 days of exercise.	693	103	707	109	Blood volume determination at end of exercise period.
	" 5 " " "	614	92	710	109	Blood volume determination at end of exercise period.
						Animal had been caged 2 wks. at the time observations were begun.
No. 16. Male mongrel; weight $10\frac{3}{4}$ kilos.	10 days before beginning exercise.	646	99	628	100	10 min. exercise before blood volume determination. Otherwise no exercise.
	2 " " " "	648	100	636	102	10 min. exercise before blood volume determination. Otherwise no exercise.
	The day exercise was begun.	660	101	610	98	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.
	After 5 days of exercise.	588	90	635	102	Blood volume determination at end of exercise period.

95 per cent of the normal. At the end of the 5th week the cell volume, as shown by the dye method, had regained the normal level, and a simultaneous CO determination gave a cell volume of 104 per cent. An extremely high plasma volume was found at this determination by both dye and CO methods.



TEXT-FIG. 1. Series A. Effect of exercise after brief confinement.

Animal 2 showed a well marked fall in cell volume when exercised after a 5 months period of confinement. Complete recovery was noted between 2 and 3 weeks later. No further exercise was given for 3 months and then the animal was subjected to 3 consecutive weeks of it. No observations were made during this period, but at



TEXT-FIG. 2. Series B. Effect of prolonged exercise in sedentary animals.

TABLE II.

Series B. Effect of Prolonged Exercise in Sedentary Animals.

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
						Animal caged 5 mos. at the time observations were begun.
No. 0. Male mongrel; weight $16\frac{3}{4}$ kilos.	4 days before beginning exercise.	670	96	960	101	No exercise.
	1 day " " "	723	104	939	99	" " Exercise begun next day. Animal exercised 4 hrs. daily for 4 days.
	After 4 days of exercise.	591	85	919	97	Blood volume determination at end of exercise period.
	19 days later.	677	97	825	87	No exercise since preceding determination.
	5 " after preceding determination.	710	102	672	71	" " before blood volume determination. Exercised 4 hrs. after blood volume determination and 4 hrs. daily for the next 3 days.
	After 4 days of exercise.	748	107	940	99	Blood volume determination at end of exercise period.
	The next day.	759	109	1,033	109	No exercise since preceding determination.
	" same "	702*	101	984*	104	" " " " "
						Animal caged for 39 days. Then exercised 4 hrs. daily 6 days in the wk. for 3 wks.
	After 3 wks. of exercise.	775	111	1,015	107	Blood volume determination at end of exercise period.
Weight at autopsy $14\frac{3}{4}$ kilos.	The same day.	774*	111	1,019*	107	Blood volume determination at end of exercise period.

						Animal had been caged 3 mos. at the time observations were begun.
No. 1. Male collie; weight 22 kilos.	6 days before beginning exercise.	1,165	94	1,090	104	No exercise.
	4 " " " "	1,270	103	975	93	" "
	The day exercise was begun.	1,276	103	1,074	103	" " before blood volume determination. Exercised 2 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days.
	After 4 days of exercise.	1,096	89	1,272	122	Blood volume determination at end of exercise period.
	" 6 " " "	1,089	88	936	89	Blood volume determination at end of exercise period.
	6 days after preceding determination.	1,425	115	1,335	128	No exercise since preceding determination.
	The same day.	1,450*	117	1,260*	120	" " " " "
	" next "	1,428	115	1,457	139	" " " " " Exercise begun the following day and animal was exercised 4 hrs. daily 4 days in that wk. and 6 days in 4 following wks.
	At end of 2nd wk. of exercise.	1,240*	100	1,140*	109	Blood volume determination at end of exercise period.
	" " " 3rd " " "	1,176*	95	1,242*	119	Blood volume determination at end of exercise period.
	" " " 5th " " "	1,288	104	1,652	158	Blood volume determination at end of exercise period.
Weight at autopsy 21½ kilos.	The same day.	1,230*	100	1,575*	151	Blood volume determination at end of exercise period.

* These determinations were made by the dye method, all others by the CO method.

TABLE II—*Continued.*

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
						Animal had been caged 5 mos. at the time observations were begun.
No. 2. Male mongrel; weight 14½ kilos.	4 days before beginning exercise.	686	96	714	96	No exercise.
	2 " " " "	682	95	710	96	" "
	The day exercise was begun.	778	109	802	108	" " before blood volume determination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.
	After 5 days of exercise.	504	71	726	98	Blood volume determination at end of exercise period.
	16 days after preceding determination.	694	97	658	89	No exercise since preceding determination.
	5 " " " "	800	112	888	120	" " " " "
						Animal caged for 3 mos., then exercised 4 hrs. daily 6 days in the wk. for 3 wks.
Weight at autopsy 14¾ kilos.	After 3 wks. of exercise.	774	108	866	117	Blood volume determination at end of exercise period.
						Animal had been caged 5 mos. at the time observations were begun.
No. 4. Female collie; weight 23¼ kilos.	8 days before beginning exercise.	1,128	98	1,084	90	No exercise.
	4 " " " "	1,148	100	1,267	105	" "
	2 " " " "	1,130	98	1,160	96	" "

	The day exercise was begun.	1,195	104	1,305	108	No exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.
	After 5 days of exercise.	834	73	978	81	Blood volume determination at end of exercise period.
	11 days after preceding determination.	960	83	840	70	No exercise since preceding determination.
	7 " " " "	1,143	99	1,127	94	" " " " "
	8 " " " "	1,107*	96	1,028*	85	" " " " "
	The same day.	1,144	100	1,066	89	" " " " "
	After 3 days of exercise.	1,065*	93	1,027*	85	Animal caged for 8 days, then exercised 2 hrs. daily for 3 days. Blood volume determination at end of exercise period. Animal rested 1 day, then exercised 4 hrs. daily 6 days in the wk. for the next 3 wks.
	7 days after preceding determination.	1,006*	87	988*	82	Blood volume determination at end of exercise period.
	5 " " " "	930*	81	1,030*	86	Blood volume determination at end of exercise period.
	The same day.	1,010	88	1,090	91	Blood volume determination at end of exercise period.
	8 days after preceding determination.	1,131*	98	1,013*	84	Blood volume determination at end of exercise period.
Weight at autopsy 22½ kilos.	The same day.	1,163	101	1,042	87	Blood volume determination at end of exercise period.

its end a determination showed the cell volume to be 108 per cent of the normal. The CO method was used throughout.

Dog 4, exercised after 5 months confinement, showed a 27 per cent decrease in cell volume at the end of 5 days. Recovery to within 99 per cent was noted on the 18th day thereafter. 8 days later a CO determination gave exactly the normal average. On the same day a dye volume determination indicated the presence of 1,107 cc. of cells, that is to say 97 per cent of the normal established by the CO determinations. The animal was caged for 8 days, then exercised for 2 hours daily during 3 days. At this time a dye volume determination showed a fall in cell volume to 93 per cent of the normal. The animal was now exercised 4 hours daily, on 6 days each week, for 3 weeks. At the end of the 1st week a dye volume determination showed a cell volume 88 per cent of the normal. 5 days later both a dye and a CO determination were made. The dye determination gave a cell volume value of 81 per cent of the normal, the CO, 88 per cent of the normal. 8 days later, determinations by both methods showed that the cell volume had returned to the normal level in spite of the continued exercise.

The Autopsy Findings.

With the exception of No. 2, the animals of Series B were killed at the end of the exercise periods and the findings in them compared with those in larger series of normal dogs, which had been caged for several months. The series of exercised animals was so small that the findings cannot be taken as more than suggestive.

The weight of the spleens was considerably greater in the exercised animals than in the controls and greater than is usual with normal dogs. Ellenberger and Baum[5] state that the spleen usually holds between $\frac{1}{500}$ and $\frac{1}{600}$ of the body weight. In ten normal animals caged for several months, which I examined, the spleen averaged $\frac{1}{441}$ of the body weight, as compared with $\frac{1}{272}$ for the three exercised animals. The differences in the amount of red marrow in the long bones of the two series of animals were marked. There was far more of it in the exercised dogs, especially near the epiphyses.

[5] Ellenberger, W., and Baum, H., Anatomie des Hundes, Berlin, 1891, 361.

DISCUSSION.

These observations on recently caged animals demonstrate that the length of the period of confinement prior to exercise determines in a measure the extent of the decrease in cell volume caused thereby. They offer further evidence on the important rôle of exercise in maintaining an active bone marrow.

The experiments on the animals of Series B show the transformation of sedentary animals into animals which are capable of withstanding severe and long continued exertion without the development of what I have termed marrow decompensation. Animal 0, exercised at a time when active regeneration was in progress, exhibited no decrease in cell volume. Animal 1, which became markedly plethoric soon after the first exercise period, sustained a loss of this plethora during subsequent exercise but the cell volume never went more than a few per cent below the normal, and the slight depression was recovered from within a week. No. 4, when exercised anew a week after the cell volume had recovered from the loss of a previous exercise period, showed again during the first 2 weeks on the treadmill a well marked decrease in cell quantity, but made this up during the 3rd week. It is highly probable that a fall in cell volume would have been found in the 1st or 2nd week of exercise on Nos. 0 and 2 when they were exercised after the second period of confinement. However, no observations were made until the end of the 3rd week and at this time both had replaced whatever loss had occurred. It is evident then that in the space of approximately 3 weeks sedentary animals can adjust themselves to meet the increased rate of blood destruction which exercise causes. These findings will explain the observation of Schneider and Havens[6] who failed to find changes in the blood volume of an athlete exercised several weeks after training had begun. No special stress can be laid on the unusually large quantity of red marrow present in the exercised animals, the autopsies being too few in number to furnish satisfactory evidence on the point. Külbs,[7] however, mentions findings which have significance in connection with those here presented. He used dogs from the same litter,

[6] Schneider, E. C., and Havens, L. C., *Am. J. Physiol.*, 1914–15, xxxvi, 239.
[7] Külbs, *Verhandl. Kong. inn. Med.*, 1909, xxvi, 197.

allowing one to remain quiet, giving another severe treadmill exercise. In three instances there was at autopsy distinctly more red marrow in the exercised dogs than in the controls. The difference is what would be expected from the physiological readjustments indicated by the present work. The demands of exercise, as expressed in terms of blood destruction, may well necessitate not merely heightened activity of the hematopoietic tissue, but a larger amount. The rôle of exercise in maintaining the hematopoietic tissue at a high level of efficiency has been pointed out in the previous paper.[3]

SUMMARY.

Animals recently caged do not show as large a decrease in cell volume after exercise as those which have been confined several months.

When a dog kept for a long time under sedentary conditions is exercised continuously for several weeks, a decrease in cell volume occurs in the 1st or 2nd week of exercise, but usually by the end of the 3rd week the loss has been made up. Apparently by this time the hematopoietic tissues have adapted themselves to the increased demands made upon them by the hastened rate of destruction. There is, furthermore, some anatomical evidence that such is the case.

A CYTOLOGICAL STUDY OF THE NATURE OF RICKETTSIA IN ROCKY MOUNTAIN SPOTTED FEVER.

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Plates 11 to 13.

(Received for publication, June 20, 1922.)

INTRODUCTION.

Conflicting views have been expressed regarding the nature of *Rickettsia* in Rocky Mountain spotted fever, typhus fever, and other diseases. While some believe that they are true microorganisms, others remain entirely unconvinced.

Strong,[1] for instance, has called attention to "the possibility that the bodies *sometimes* described as *Rickettsia* may constitute products of degenerated cells, for example, basophilic granules, which are more numerous in the blood in certain febrile diseases, in which case they would also increase in number in lice fed upon such cases, and might then merely accompany the very minute or invisible ætiological factor of the disease." Woodcock[2] advanced the suggestion that *Rickettsia* in mammals are merely endothelial cell inclusions of phagocytosed hemoglobin particles, but he was unwilling at the time to commit himself to a similar interpretation of the *Rickettsia* seen in the tissues of insects and arachnids, although in a later paper he has apparently done so.[3] His contention merits careful consideration on account of the extensive destruction of red blood corpuscles which is known to occur in Rocky Mountain spotted fever, in which disease Wilson and Chowning[4] found as many as eight phagocytosed erythrocytes within a single cell. Stevenson and Balfour[5] have expressed themselves with considerable reserve concerning the identification of *Rickettsia* in human tissues taken from cases of typhus fever, as distinct from pigment granules of hematogenous origin.

[1] Strong, R. P., Shattuck, G. C., Sellards, A. W., Zinsser, H., and Hopkins, J. G., Typhus fever with particular reference to the Serbian epidemic, Cambridge, 1920.

[2] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1921, xxxvii, 418.

[3] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1922, xxxix, 251.

[4] Wilson, L. B., and Chowning, W. M., *J. Am. Med. Assn.*, 1902, xxxix, 131.

[5] Stevenson, A. C., and Balfour, A., *J. Path. and Bact.*, 1921, xxiv, 289.

There are several points in Wolbach's[6] description of the *Rickettsia* of Rocky Mountain spotted fever which have given rise to the suggestion that, in mammals, he may have been dealing with mitochondria. In the first place, the striking, though superficial, morphological resemblance between the *Rickettsia* and mitochondria is to be noted, and it will be recalled in this connection that the general similarity between bacteria and mitochondria has recently led Wallin[7] to express the belief that mitochondria are in reality bacteria which have developed a high degree of symbiosis. Then the recommendation that acetic acid be omitted in making up the Zenker's fluid for the purpose of preserving the *Rickettsia*, with the admission that Schaudinn's mixture, consisting of two parts of saturated corrosive sublimate with one part of absolute alcohol, does not demonstrate them satisfactorily, points in the same direction. And finally, the failure of all investigators to cultivate them artificially, coupled with his almost complete silence on the subject of mitochondria, has tended to strengthen the impression thus created. The only reference which he has made to this possibility of confusion with mitochondria is contained in a more recent paper on typhus fever written in collaboration with Todd and Palfrey,[8] in which it is recorded that the Zenker's fluid was made up with 5 per cent of acetic acid "in order to prevent the staining of mitochondria, although slightly better results may be obtained without it." Considered alone this evidence is not wholly convincing, because under certain conditions they are not dissolved by the acetic acid in Zenker's fluid.[9]

It was in the belief that the exact relation of the organisms to normal and abnormal cellular constituents might be susceptible to analysis by methods elaborated in the field of general cytology that the study herein reported was undertaken. It was made possible by the kindness of Dr. Noguchi, who furnished a generous supply of tissues from guinea pigs infected with Rocky Mountain spotted fever.

Material and Methods.

The pathological tissues and the specimens of *Dermacentor venustus* used in these experiments formed a portion of the material of Dr. Noguchi, who for several years has been engaged in experimental studies of spotted fever. The maintenance of the virus and its

[6] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 1.

[7] Wallin, I. E., *Am. J. Anat.*, 1922, xxx, 203.

[8] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

[9] Cowdry, E. V., and Olitsky, P. K., *J. Exp. Med.*, 1922, xxxvi, 521.

transmission by ticks were carried out by Dr. Noguchi, with suitable clinical and bacteriological controls to eliminate secondary bacterial infections, and all autopsies were performed by Dr. Noguchi and his staff. The two strains of spotted fever virus, as well as the specimens of wood ticks used in this work, were obtained by Dr. Noguchi through the courtesy of Dr. R. R. Parker of Hamilton, Montana.

Autopsies were made and tissues taken 1, 2, 3, 4, and 5 days after the 1st day of characteristic fever. In practically every animal tissues from the sole, scrotum, testes, lymph glands, kidneys, adrenals, pancreas, liver, spleen, abdominal muscle, lungs, heart, and ears were carefully examined. In addition to one normal guinea pig, three others, which had recovered from the infection, were studied for purposes of control.

For the coloration of *Rickettsia*, Giemsa's stain was used, as recommended by Wolbach, except that the preliminary fixation was accomplished in Regaud's fluid instead of in Zenker's mixture. This fluid consisted of four parts of a 3 per cent solution of potassium bichromate and of one part of commercial formalin. It gave a clear background with but little artificial granulation and at the same time acted as a good preservative for mitochondria. For a comparison of *Rickettsia* with normal and abnormal cellular constituents, the aniline-fuchsin and methyl green method of Bensley was employed for mitochondria,[10] the iron-hematoxylin technique for phagocytosed hemoglobin,[10] and Kyes' method for Gram-positive organisms in tissues.[11]

Several preparations of the *Rickettsia* of Rocky Mountain spotted fever in the tissues of ticks and guinea pigs, kindly donated by Professor Wolbach, were used for comparison.

OBSERVATIONS.

Wolbach's discovery that *Rickettsia* of somewhat similar morphology occur in infective ticks and in the specific lesions of guinea pigs suffering from Rocky Mountain spotted fever contracted through

[10] Mallory, F. B., and Wright, J. H., Pathological technique, Philadelphia and London, 7th edition, 1918.

[11] Kyes, P., personal communication.

their bites was soon amply confirmed. The former are illustrated in Figs. 18 and 19 and the latter in Fig. 17.

Preparations were made which showed that, unlike the mitochondria, they are restricted to the vascular lesions peculiar to the disease, in which the mitochondria are definitely reduced in number and changed in shape. Though it was found that the *Rickettsia* may be stained by both the iron-hematoxylin and the aniline-fuchsin and methyl green techniques, that is to say by two methods which are primarily intended for mitochondria, and that when appropriately mordanted the mitochondria may sometimes be colored by Giemsa's stain, in both cases the color reactions were so atypical that they could not be taken to indicate the existence of a close degree of similarity.

After fixation in Regaud's fluid, it was possible to color the *Rickettsia* and the mitochondria successively in the same cells and to demonstrate their independence. Fig. 1 illustrates the morphology and distribution of the *Rickettsia* in a typical venule of a guinea pig during the 5th day of fever, as brought out by staining by Giemsa's method. Fig. 2 represents the mitochondria (which have lost their rod-like and filamentous shape) in the same cells after the section had been decolorized and stained with aniline-fuchsin and methyl green. A close comparison of the two will reveal further points of distinction.

The claim that *Rickettsia* may be products of the phagocytosis of injured erythrocytes, or of nuclear degeneration, was tested by decolorizing the same section, once more, and by staining it with iron-hematoxylin (Fig. 3). It will be seen that the areas in which *Rickettsia* have been shown to occur (Fig. 1) remain entirely clear by this method, and, conversely, that blackened materials occur in this figure in places where they are not seen in Fig. 1.

It may therefore be concluded, from Figs. 1, 2, and 3, that the *Rickettsia*, the altered mitochondria, and the products of the phagocytosis of injured erythrocytes and of endothelial cell degeneration, though frequently occurring side by side, are entirely different in respect to their tinctorial properties, their morphology, and their distribution.

Although the *Rickettsia* were found to be colored in much the same

way as certain amphophilic granules, which had escaped from leucocytes and become distributed through the tissues, they were easily distinguished from the amphophilic granules by their rod-like shape and by their characteristic tendency to accumulate within the cytoplasm of endothelial cells. Neither is it possible to confuse them with the granules which Wolbach[8] has referred to as arising in endothelial cells in stages preliminary to division.

The only other normal constituent of endothelial cells with which there is a possibility of confusing *Rickettsia* is the internal reticular apparatus of Golgi. From the descriptions of von Bergen[12] and others relating to its appearance in endothelial cells it is safe to conclude that it occurs in the form of a rather complicated network of coarse strands situated at one or the other pole of the nucleus. In certain pathological conditions in which it has been studied, the apparatus undergoes disintegration[13] with the liberation of innumerable tiny droplets of material which impregnate with silver salts and blacken with osmic acid. These reactions are entirely different from those of the *Rickettsia*, and, conversely, the internal reticular apparatus is never colored with Giemsa's stain.

Evidence having thus been obtained pointing to the conclusion that *Rickettsia* are entirely distinct from all known cellular components, their distribution was carefully studied in the tissues of guinea pigs. A list of the animals examined with the tissues in which they were found follows.

1 day of fever: adrenal and epididymis.
1 day of fever: pancreas.
2 days of fever: abdominal muscle.
3 days of fever: abdominal muscle, scrotum, testis, and heart.
3 days of fever: testis, spleen, lung, and abdominal muscle.
4 days of fever: scrotum and testis.
4 days of fever: scrotum and heart.
5 days of fever: scrotum, testis, lymph gland, liver, spleen, lung, and abdominal muscle.
5 days of fever: scrotum and testis.

A rather wide distribution of *Rickettsia* was anticipated from Wolbach's account of their presence in the skin, scrotum, and epididy-

[12] von Bergen, F., *Arch. mikr. Anat.*, 1904, lxiv, 498.
[13] Penfield, W. G., *Brain*, 1920, xliii, 290.

mis of guinea pigs, rabbits, and monkeys, and in the thyroid, testis, liver, spleen, lungs, and skeletal muscle, in addition to these locations, in man and the monkey. From the present study their dispersal in guinea pigs was extended to include, in addition, the pancreas, abdominal muscle, adrenals, heart, lymph glands, lung, testis, liver, and spleen (Figs. 1, 5 to 7, 9 to 12, and 14).

The *Rickettsia* were observed in all guinea pigs known to be infected and were found to be uniformly absent in the control as well as in three animals which recovered from the infection. They were invariably associated with the characteristic vascular lesions described in the literature, and increased in number progressively with the development of the lesions from the 1st to the 5th or last day of fever, when both attained a maximum.

Their occurrence for the most part in the endothelial and smooth muscle cells of blood vessels and occasionally in skeletal muscle cells, as described by Wolbach, was confirmed and extended to include the vascular lumina, one giant cell, a few liver cells, and mononuclear leucocytes, as illustrated in Figs. 1, 8, 12, and 1. In several cases also, they appeared to be present within erythrocytes (Fig. 13) and in one instance within the nucleus of an endothelial cell (Fig. 5). They were never quite so abundant as represented by Wolbach.[14] In some tissues it required several hours search with the aid of a mechanical stage and in others a day or more to find them. Sections more than 5 microns in thickness were found to be useless, owing to the fact that the longer differentiation required bleached them out completely.

The arrangement of *Rickettsia* within the cytoplasm of endothelial cells appeared to be haphazard. Sometimes they were observed singly and at other times in groups (Figs. 14 and 15) without definite topographical relation to the nuclei, to areas containing a larger number of mitochondria, or to the proximity of phagocytosed hemoglobin, as would be expected in cells possessing so little physiologic polarization. This independence may best be observed by comparing Figs. 1, 2, and 3. With the exception of the liver they were not found in parenchymatous cells, which exhibit a more marked division into areas of differential functional activity. Neighboring endothelial

[14] Wolbach,[6] Figs. 46, 66, and 68.

cells containing many *Rickettsia* and others containing none at all had much the same swollen appearance (Fig. 1). In other words the degree of injury in contiguous cells was not paralleled by the number of enclosed *Rickettsia*. That the mitochondria in all the endothelial cells bordering an affected blood vessel were about equally altered may be seen by comparing Fig. 2 with Fig. 4, which illustrates their normal appearance. The chief change in the mitochondria was a great diminution in number and a rounding up of the survivors into spheres, with slight increase in size and a loss of their original rod-like and filamentous shape. This type of mitochondrial reaction has been described frequently in the literature following cellular injuries of different kinds; it is in no sense peculiar to Rocky Mountain spotted fever.

During the first 2 days of fever the chromatin content of the nuclei of the endothelial cells appeared to be decreased (Figs. 5 and 6), about the 3rd day it seemed increased (Fig. 7), only to decrease again during the 4th and 5th days (Fig. 17), but owing to the difficulty of making accurate determinations it is felt that a definite statement is not warranted. No parallel oscillations were noted in the *Rickettsia*, in the mitochondria, in the quantity of phagocytosed material, or in the rate of cellular multiplication, evidence of which was seen in occasional mitoses.

Early in the infection the *Rickettsia* were diplobacillary (Fig. 16), becoming bacillary in the later stages (Fig. 17). Their ends were rounded rather than lanceolate. Coccal and diplococcal forms comparable to those illustrated by Wolbach[15] and others which were seen in his preparations were not observed in my material.

Tinctorially, the *Rickettsia* in guinea pig tissues reacted in a homogeneous way without showing evidence of polar staining. In all cases they were colored dark reddish brown, the red tint in which could be altered to some extent by varying the amount of colophonium in the alcohol of differentiation as suggested by Wolbach. There was but little variation in the intensity of coloration of individual *Rickettsia*. All of them were Gram-negative. In some instances they were surrounded by very slight halos of chromophobe material, which, however, never attained the dimensions represented by Wolbach. In some of

[15] Wolbach,[6] Figs. 66 and 68.

his illustrations the halos are inconspicuous, or entirely absent,[16] while in others[17] they exceed in thickness the diameter of the *Rickettsia*. Variations of this magnitude were not observed in the present study. A close examination of his preparations revealed the fact that the contained *Rickettsia* were stained much bluer than in my own and that their outlines were considerably less distinct. This indistinctness he mentioned as a non-bacterial characteristic.

These diplobacillary and bacillary forms observed in the lesions of guinea pigs seem to correspond in part at least to the first stage of Wolbach's morphologic series which he characterized as follows: "An extra-nuclear bacillus-like form without chromatoid granules, relatively large and only present in ticks during the initial multiplication of the parasites."

Similar but rather more pleomorphic *Rickettsia* were found in the tissues of infective ticks as may be seen by comparing Figs. 18 and 19 with Figs. 16 and 17. Some of the smaller ones were stained more intensely at the ends (Fig. 19), but none were detected within the nuclei, and it was not possible, in the material available, to recognize clearly the three morphologic types enumerated by Wolbach. Close study of his actual preparations brought to light many exceedingly minute rod forms with chromatoid granules, which, as in the case of mammalian tissues, stained bluer and presented less distinct outlines than the *Rickettsia* in my own specimens.

SUMMARY.

The *Rickettsia* of Rocky Mountain spotted fever were easily differentiated from mitochondria, phagocytosed blood pigment, nuclear debris, and all other known cellular constituents. Although they were lodged within the cytoplasm of endothelial cells, they were not observed to establish any definite relations with the nucleus or with other cellular components. Their number varied in contiguous cells which sustained the same degree of injury as evidenced by nuclear changes, and alterations in their mitochondria content. The mitochondria, on the other hand, showed similar modifications, character-

[16] Wolbach,[6] Fig. 48.
[17] Wolbach,[6] Fig. 68.

ized by a decrease in number and a rounding up into spherules, in all the endothelial cells seen in a section of an affected blood vessel. Diplobacillary forms were most abundant in the early stages of the reaction and single bacillary bodies towards its termination. Other slight differences in morphology from Wolbach's account were noted in the organisms as seen in the tissues of both ticks and guinea pigs. His study of the distribution of specific lesions with accompanying organisms in the tissues of guinea pigs was confirmed and extended.

EXPLANATION OF PLATES.

All the illustrations were drawn with Zeiss apochromatic objective 1.5 mm., compensating ocular 6, and camera lucida. Unless otherwise stated, the preparations were fixed in Regaud's fluid and colored with Giemsa's stain. Magnification, × 1,500.

PLATE 11.

FIG. 1. Venule from the abdominal wall during the 5th day of fever showing *Rickettsia* within the lumen as well as in two large mononuclear leucocytes and in some of the endothelial cells.

FIG. 2. The same tissue after decoloration and staining with aniline-fuchsin and methyl green which has colored the mitochondria.

FIG. 3. The same after a second decoloration and staining with iron-hematoxylin which has blackened the nuclear chromatin and normal and phagocytosed red blood cells.

FIG. 4. Venule from a normal guinea pig stained with aniline-fuchsin and methyl green to illustrate the normal appearance of mitochondria.

PLATE 12.

FIG. 5. Venule in the pancreas during the 1st day of fever showing *Rickettsia* within the nucleus of an endothelial cell from which most of the chromatin has disappeared.

FIG. 6. Venule in the adrenal gland during the 1st day of fever containing scattered *Rickettsia*.

FIG. 7. Venule in the heart during the 3rd day of fever showing scattered diplobacillary *Rickettsia*.

FIG. 8. Giant cell in the scrotum during the 5th day of fever containing many clumped bacillary *Rickettsia*.

FIG. 9. Lymph gland capillary during the 5th day of fever with scattered *Rickettsia*.

FIG. 10. Venule in the lung during the 5th day of fever with diplobacillary and bacillary *Rickettsia*, the latter predominating.

FIG. 11. Capillary in the spleen during the 5th day of fever with diplobacillary and bacillary *Rickettsia*.

FIG. 12. Sinus in the liver during the 5th day of fever with two *Rickettsia* in the nucleus (upper right hand corner), one within the lumen, and three others in the cytoplasm of a liver cell facing the lumen.

FIG. 13. The same showing one *Rickettsia* apparently within an erythrocyte.

FIG. 14. Venule in the testis during the 5th day of fever with *Rickettsia* definitely clumped.

FIG. 15. The same.

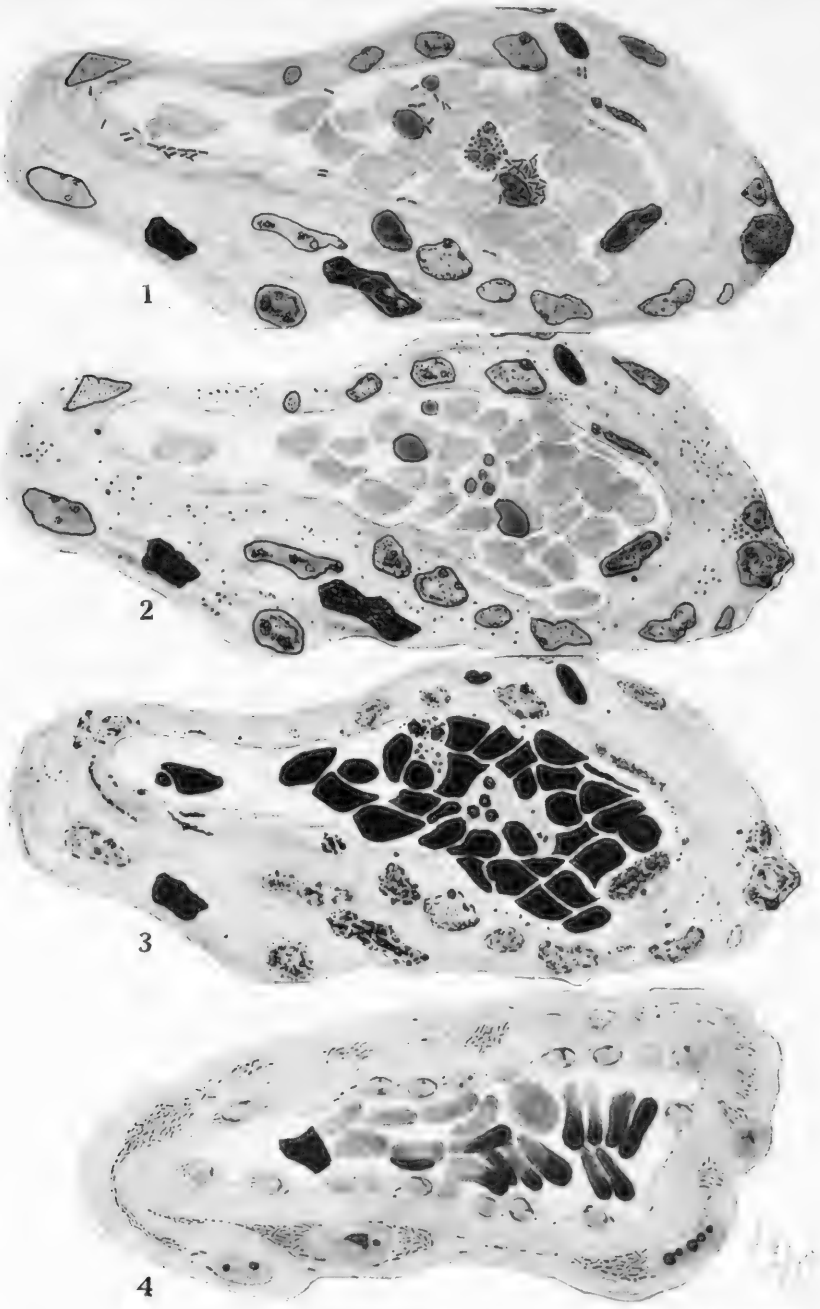
PLATE 13.

FIG. 16. Venule in a seminal vesicle during the 1st day of fever containing for the most part diplobacillary *Rickettsia*.

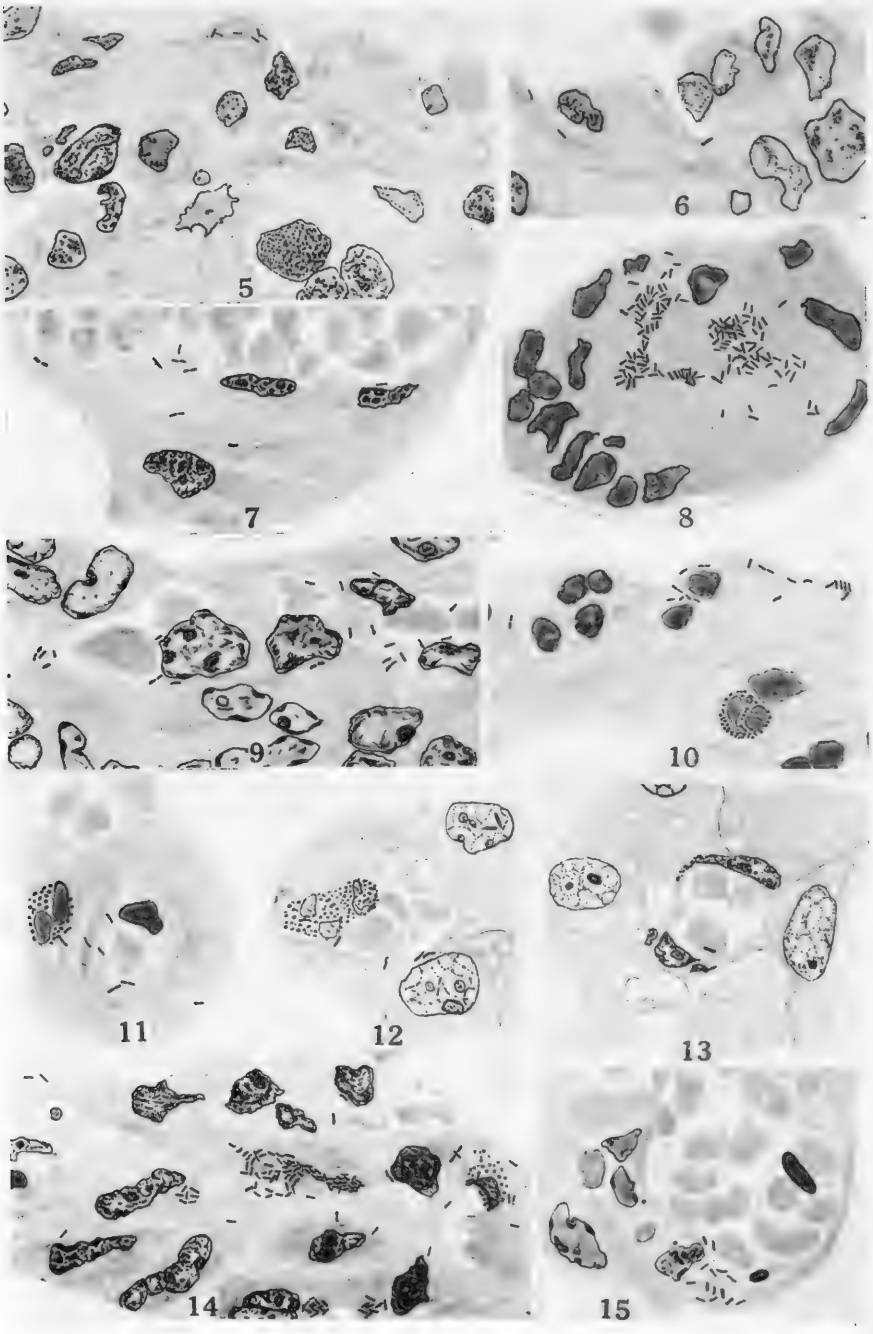
FIG. 17. Venule in the scrotum during the 5th day of fever containing bacillary forms.

FIG. 18. Ovarian epithelium of *Dermacentor venustus* containing *Rickettsia* which are rather more pleomorphic.

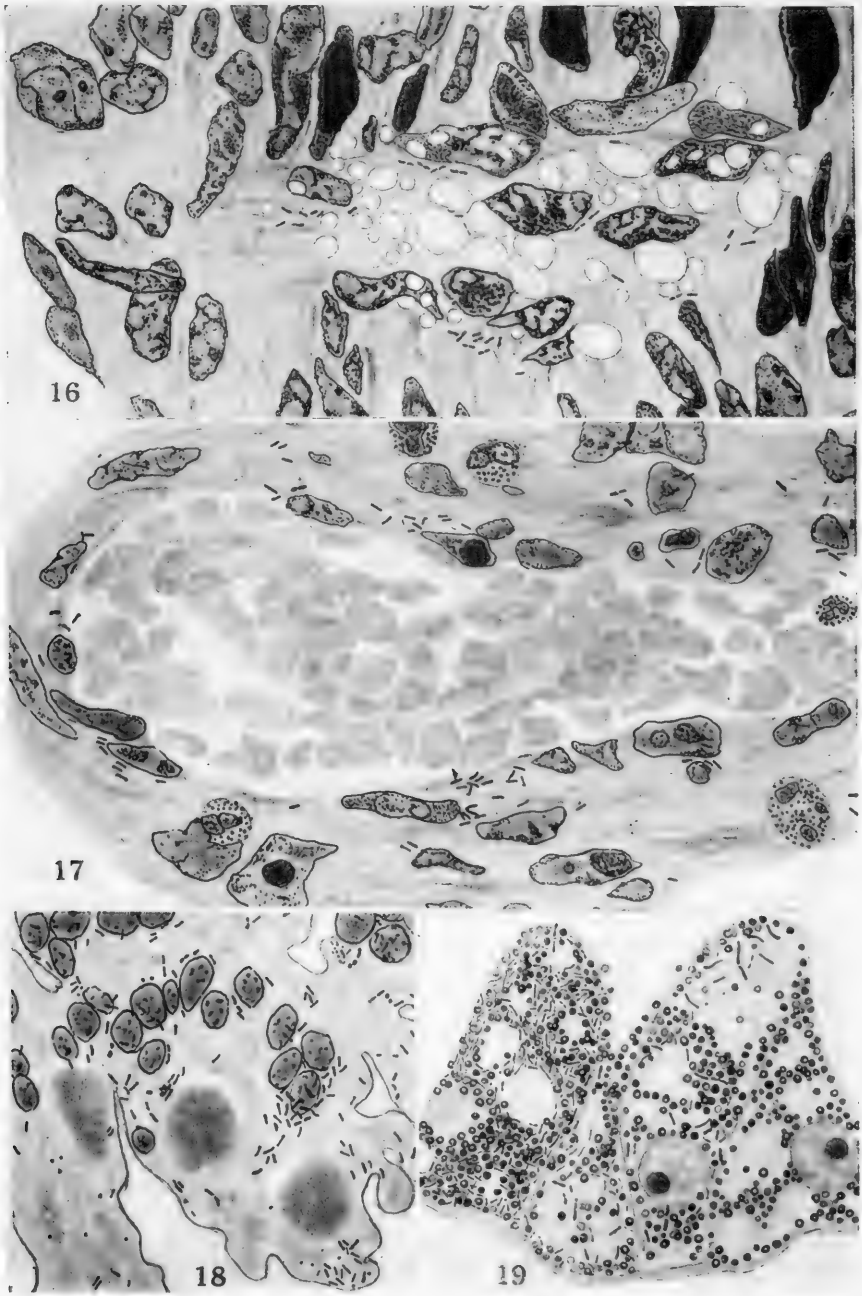
FIG. 19. Intestine of *Dermacentor venustus* showing similar *Rickettsia*.



(Nicholson: *Rickettsia* in Rocky Mountain spotted fever.)



(Nicholson: *Rickettsia* in Rocky Mountain spotted fever.)



(Nicholson: *Rickettsia* in Rocky Mountain spotted fever.)

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN MOUSE TYPHOID INFECTION.

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(Received for publication, August 25, 1922.)

An experimental epizootic of mouse typhoid caused by a member of the paratyphoid-enteritidis group, *Bacillus pestis caviæ*, has been studied by Flexner and Amoss at The Rockefeller Institute.[1,2,3] At the same time a number of tests were devised with various factors controlled or kept constant to analyze some of the observed phenomena.[4] It was shown that partial or complete protection of a general nature followed the administration of killed or living cultures intrapleurally, intraperitoneally, or *per os*. Also it appeared that after pleural or peritoneal injection, duration of life and type of disease were accurately related to dosage but that if the cultures were administered *per os*, duration of life was not so characteristically a function of dosage. Regardless of the number of organisms ingested, some animals proved refractory to infection by the gastrointestinal route, the normal portal of entry.

This latter observation would indicate that mice vary in their susceptibility to infection by *Bacillus pestis caviæ*; it throws light on the fact that throughout the course of mouse typhoid epizootics a certain percentage of animals survives, and it offers an admirable starting point for the study of disease as it occurs under natural conditions.

Sporadic infection as well as epidemic infection is concerned with three general variables, the distribution of the microbe, the virulence of the microbe, and the susceptibility of the host. With the first

[1] Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

[3] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 45.

[4] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

factor, the distribution and spread of the etiological agent, we shall not be concerned at present. But after the pathogenic organism has reached in proper quantity the usual portal of entry of its normal host, sporadic and epidemic infection will depend upon the balance between the second and third factors, microbic virulence and host susceptibility.

The following experiments, then, are an attempt to analyze the problems of infection by studying this equilibrium between microbic and host potentialities.

General Procedure.

Series of mice were injected *per os* by stomach tube with fixed doses of *Bacillus pestis caviæ*. At short intervals thereafter, the condition of the animals was noted, stools and blood were examined for the presence of the ingested microbe, the general character of the fecal flora was noted, and blood was tested for homologous agglutinins.

Technique.

White mice of uniform age and weight, obtained from the stock breeding room, were brought to the laboratory and maintained on the usual diet of bread and milk. To eliminate the contact factor and a possible reingestion of bacilli each animal was placed in a separate jar, strict cleanliness was observed in feeding and handling, and the jars were sterilized every week. Preliminary examination of blood and feces for bacteria of the paratyphoid-enteritidis group was always negative; in no cases were blood agglutinins demonstrable.

The strain of *Bacillus pestis caviæ* associated with the experimental epizootics[1,2,3] and employed in our earlier work[4] was used throughout these tests.

Stool cultures were examined in the following manner. The specimen of feces removed with care about 2 hours after feeding was emulsified with 5.0 cc. of salt solution in a sterile mortar. 0.2 cc. of this suspension was spread over a 14 cm. Petri dish containing plain agar plus 1 per cent dextrose. After 24 hours incubation the general character of the flora was noted and suspicious colonies were inoculated into plain broth. 24 hours later, agglutination tests were set up with

a formalinized broth suspension and immune rabbit serum with a titer of 1:20,000. Flocculation in a dilution of 1:10,000 was considered a sufficient criterion for the identity of the strain.[5]

It must be remembered that while a positive stool culture is reliable, a single negative test has little significance; at least three negative examinations are requisite for any definite conclusion.

Blood cultures were treated as follows: 0.02 cc. of blood from the tail vessels was spread over a dextrose agar Petri dish. After 24 hours incubation the plates were found either to be sterile or to contain the ingested microbe, usually in pure culture. Nevertheless, frequent agglutination tests were run as controls. The solid medium was found to be slightly more sensitive than fluid medium. Then, too, it allowed opportunity for rough quantitative estimates.

The amount of blood removed, 0.02 cc., corresponds to about $\frac{1}{100}$ of the total volume. This method is, therefore, about twice as accurate as that usually employed in human typhoid cases, and negative results indicate that very few if any organisms are circulating in the blood stream at that time.

Agglutination tests were performed about as described by Amoss.[3] A formalinized antigen was used; dilutions began at 1:20 and were doubled as far as 1:20,480.

Preliminary Experiments.

Experiment 1.—14 mice weighing 18 gm. each were placed in separate jars. On Mar. 13, 17, and 21, 1922, stool cultures showed no organisms of the paratyphoid-enteritidis group. On Mar. 22, agglutination tests were negative in dilutions of 1:20 and 1:40.

Mar. 22. The mice were given by stomach tube an 18 hour culture of *B. pestis caviæ* in a dilution of 1:100. This strain had been used frequently in other tests and was kept in stock on a plain agar stab.

At short intervals feces and blood were examined for the ingested organism, the general character of the flora was noted, the blood was tested for homologous agglutinins, and the condition of the animal was noted.

Individual protocols show the course of events (Table I).

It appeared immediately that the animals might be divided into at least three general groups, as follows: (1) Those which

[5] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

TABLE I.

Protocols of the Mice Used in Experiment 1.

Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutinins.	Condition of animal.
	1922					
1	Mar. 21	A, few C*	0	——	0	Well.
	" 22	Bacilli ingested.				
	" 23	A, few C	0	0	0	Well.
	" 25	" " "	0	0	0	"
	" 27	" " "	0	0	0	"
	" 29	" " "	0	+ (1 col.).	0	"
	" 31	" " "	0	+	0	"
	Apr. 3	——	——	++	——	"
	" 6	C = A	0	+ (1 col.).	0	"
	" 12	" = "	+	0	0	"
	" 18	A, few C	0	0	——	"
	" 25	" " "	0	0	1:20 to 1:640 ++†	"
	May 9	" " "	0	0	1:20 to 1:640 ++†	"
	" 17	——	——	——	1:20 to 1:5,120 ++ 1:10,240−	"
	" 22	A, few C	0	0	——	"
2	Mar. 21	" " c	0	——	0	"
	" 22	Bacilli ingested.				
	" 23	A, few C	+	0	0	Well.
4	Apr. 3	A	0	0	——	Sick.
	" 6	" = C	0	+ (1 col.).	——	"
	" 12	" = "	0	+ (1 ").	1:20 to 1:40 ++†	"
	" 18	" = "	++	0	——	Fair.
	" 25	" = "	0	0	1:20 to 1:640 ++†	Well.
	May 9	" few "	0	0	1:20 to 1:10,240 ++†	"
	" 17	——	——	——	1:20 to 1:10,240 ++ 1:20,480−	"
	" 22	A, few C	0	0	——	"
5	Mar. 21	" = "	0	——	0	"
	" 22	Bacilli ingested.				
	" 23	A = C	++	0	0	Well.
	" 25	" few " and c	+	0	0	"
	" 27	A = C	+	++	0	Sick.
	" 28	——	——	——	——	D.‡§
6	" 21	A = C	0	——	0	Well.

	Mar. 25	A, few c	0	0	0	Well.		Mar. 22	Bacilli ingested.				
	" 27	" " "	0	0	0	"							
	" 29	" " "	0	0	0	"		" 23	A = C	++	0	0	Well.
	" 31	" " C and c	0	0	0	"		" 25	" = "	+	0	——	"
								" 27	" = "	++	0	0	"
	Apr. 3	A, few C and c	0	0	——	"		" 29	"	0	+	0	Sick.
								" 31	C, few A and c	+	+	0	"
	" 6	A	0	0	0	"							
	" 12	C	0	0	0	"		Apr. 3	C = A	0	0	——	"
	" 18	A, few C	0	0	0	"		" 6	" = "	0	+ (1 col.).	0	Fair.
	" 25	" " "	0	0	0	"		" 12	" few "	++	0	0	"
	May 9	" " "	0	0	0	"		" 18	"	0	0	——	"
	" 22	" " "	0	0	——	"		" 25	——	——	0	0	"
								May 9	——	——	0	1:20 to 1:40 ++†	"
3	Mar. 21	" " "	0	——	0	"							
	" 22	Bacilli ingested.						" 16	——	——	——	——	D. ‡ §
	" 23	A, few C	++	0	0	Well.	7	Mar. 21	A, few c	0	——	0	Well.
	" 25	C " A	0	0	0	"		" 22	Bacilli ingested.				
	" 27	" " "	++	++	0	Sick.							
	" 28	——	——	——	——	D. ‡ §		" 23	A	0	0	0	Well.
								" 25	C, few A	0	0	0	"
4	" 21	A = C	0	——	0	Well.		" 27	A	++	0	0	"
	" 22	Bacilli ingested.						" 29	" few C	++	0	0	"
								" 31	" " "	++	0	0	"
	" 23	A = C	0	0	0	Well.		Apr. 3	" " "	0	0	——	"
	" 25	" = "	0	0	0	"		" 6	" " "	++	0	0	"
	" 27	" = "	0	0	0	"		" 12	" " "	0	0	0	"
	" 29	——	——	+	0	Sick.		" 18	"	0	0	——	"
	" 31	A = C	+ (1 col.).	++	——	"		" 25	" few C	0	0	0	"
								May 9	——	——	——	——	D. ‖

* C indicates colon colonies; c, coccus colonies; A, *acidophilus* colonies; ——, test not performed; 0, test negative; +, 1 to 10 colonies; ++, 10 to 50 colonies; +++, more than 50 colonies; —, test negative. † Further dilutions not made. ‡ Autopsy lesions typical of mouse typhoid. § Heart's blood culture positive. ‖ No lesions. Heart's blood negative.

TABLE I—*Continued.*

Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutinins.	Condition of animal.
	1922					
8	Mar. 21	A, few c	0	——	0	Well.
	" 22	Bacilli ingested.				
	" 23	A, few c	0	0	0	Well.
	" 25	" " "	0	0	0	"
	" 27	" " C and c	++	++	0	Sick.
	" 28	——	——	——	——	D.‡§
9	" 21	A, few C and c	0	——	0	Well.
	" 22	Bacilli ingested.				
	" 23	A, few c	0	0	0	Well.
	" 25	" " "	0	0	0	"
	" 27	" " "	0	0	0	"
	" 29	" " "	0	0	0	"
	" 31	" = C	++	0	0	"
	Apr. 3	——	——	0	——	"
	" 6	A	0	0	0	"
	" 12	"	0	0	0	"
	" 18	——	——	0	——	"
	" 25	A, few C	0	0	0	"
	May 9	" " "	0	0	0	"
	" 22	" " "	0	0	——	"
11	Apr. 12	A	0	0	0	Well.
	" 18	" few C	0	0	——	"
	" 25	" = "	0	0	0	"
	May 9	" few "	0	0	0	"
	" 22	" = "	0	0	——	"
12	Mar. 21	" few c	0	——	0	"
	" 22	Bacilli ingested.				
	" 23	A, few c and C	++	0	0	Well.
	" 25	" = C = c	+	0	0	"
	" 27	A, few c " " "	0	0	0	"
	" 29	"	0	+	0	"
	" 31	" few c	0	+	0	"
	Apr. 3	"	0	+	——	Fair.
	" 6	" few C	0	+	0	Sick.
	" 12		+	0	1:20 to 1:40 ++†	Fair.
	" 18	" = " ——	0	0	——	"
	" 25		——	0	1:20 to 1:640 ++†	"
	May 9	A, few C	0	0	1:20 to 1:10,240 ++	Well.
	" 22	" " " and c	0	0	1:20,480 —	"

10	Mar. 21	A = C	0	—	0	Well.
	" 22	Bacilli ingested.				
	" 23	A, few c	0	0	0	Well.
	" 25	" " "	0	0	0	"
	" 27	" " "	++	0	0	"
	" 29	" " "	0	0	0	"
	" 31	" " "	0	0	0	"
	Apr. 3	" = C	0	0	—	"
	" 6	"	0	0	0	"
	" 12	" few C	0	0	0	"
	" 18	" " "	0	0	—	"
	" 25	" " "	0	0	0	"
	May 9	" = "	0	0	0	"
	" 22	" = "	0	0	—	"
11	Mar. 21	" few c	0	—	0	"
	" 22	Bacilli ingested.				
	" 23	A, few c	0	0	0	Well.
	" 25	" " "	0	0	0	"
	" 27	" " "	0	0	0	"
	" 29	" " "	0	0	0	"
	" 31	" " "	0	0	0	"
	Apr. 3	"	0	0	—	"
	" 6	"	0	0	0	"
13	Mar. 21	A = C	0	—	0	Well.
	" 22	Bacilli ingested.				
	" 23	A = C	0	0	0	Well.
	" 25	C, few A	+	0	0	"
	" 27	" " "	+	0	0	"
	" 29	" = "	0	0	0	"
	" 31	" = "	++	+	0	"
	Apr. 3	A, few C	0	0	—	"
	" 6	"	0	0	0	"
	" 12	"	0	0	0	"
	" 18	"	0	0	—	"
	" 25	"	0	0	0	"
	May 9	" few C	0	0	0	"
	" 22	"	0	0	—	"
14	Mar. 21	" few c	0	—	0	"
	" 22	Bacilli ingested.				
	" 23	A, few c	0	0	0	Well.
	" 25	" = C	—	+ (1 col.).	0	Sick.
	" 27		—	0	0	"
	" 29		—	+	0	"
	" 31		—	++	—	"
	Apr. 3		—	++	—	"
	" 4		—	—	—	D.§

either destroyed the bacilli at once or passed them with the feces for a period of time. Blood cultures and agglutination tests from these animals were persistently negative. The mice remained quite normal throughout the 8 weeks observation. (2) A group which, besides passing the bacilli with the stools for an irregular period of time, showed small numbers of specific organisms in the blood stream for 2 or 3 weeks. Agglutinins of high titer appeared after 3 weeks; the animals, after showing typical symptoms of mouse typhoid, recovered completely. (3) A third group which passed bacilli in large numbers with the stools, showed positive blood cultures, and, after the usual incubation period of 5 days, succumbed in varying intervals of time.

Mouse 13, which had a few bacilli in the blood on one occasion but no agglutinins, was a connecting link between Groups 1 and 2; similarly, Mice 6 and 14, which succumbed after the appearance of agglutinins, might be placed between Groups 2 and 3.

This experiment, then, analyzed the wide variations in host susceptibility to a fixed dose of *Bacillus pestis caviæ*.

It is fair to assume that during any epidemic the virus in question is at its highest level of virulence, that a vigorous strain has been selected which is well adapted to the biological medium of the host. It was therefore desirable that the virulence of the strain should be at its maximum, so that phenomena subsequent to its ingestion would be analogous to those occurring during an experimental epizootic of mouse typhoid. A rapid series of animal passages *per os* was then conducted with this aim in view.

Also, throughout this work, the strain of *Bacillus pestis caviæ*, isolated from the first animal to die in the above series of animal passages, has been transferred continually from series to series in the following manner. An 18 hour heart's blood broth culture diluted 1:200 is injected by stomach tube into at least ten mice weighing about 16 to 18 gm. each. The first animal to die is then autopsied and the heart's blood culture, after proper precautions, is injected into the next series, as described above.

Experiment 2 brings together for comparison and study, as Experiment 1, three series of normal mice injected *per os* with the active strain.

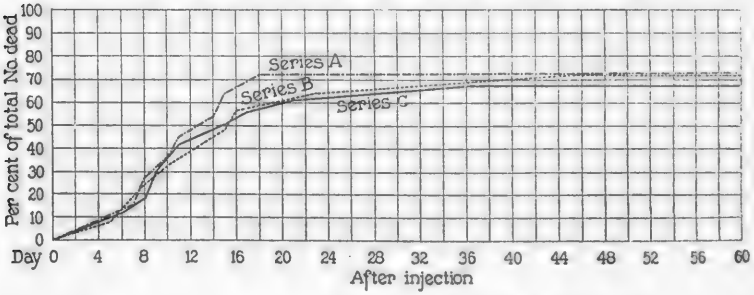
Experiment 2.—Series A: 16 normal mice from the breeding room, weighing about 18 gm. each, were placed in separate jars. The regular diet of bread and milk was continued. The animals were then given by stomach tube an 18 hour heart's blood broth culture, diluted 1:100, of the active strain from the first mouse to die in a routine passage series. Blood cultures were not taken.

Series B: 1 month later 12 normal mice from the breeding room were treated in the same way. Results of stool and blood cultures, agglutinin reactions, and condition of the animals were recorded.

Series C: 1 week later 11 normal mice from the breeding room were injected and studied in a similar manner.

Text-fig. 1 shows the mortality curves of the three series.

About 30 per cent of the mice in each series survived. They all belonged to Group 1, which passed the bacilli for a few days with the stools but showed no positive blood cultures and no agglutinins. They remained well throughout the entire 8 weeks of observation.



TEXT-FIG. 1. Mortality curves of the mice used in Experiment 2.

The striking similarity of the mortality curves (Text-fig. 1) leads one to regard them as being more or less constant under the given conditions; that is, as representing the balance between microbic virulence and collective host susceptibility. If reliable, they may explain many phenomena of experimental mouse typhoid epizootics and may change not a few unknown variables to constants in the mathematical study of epidemiology.

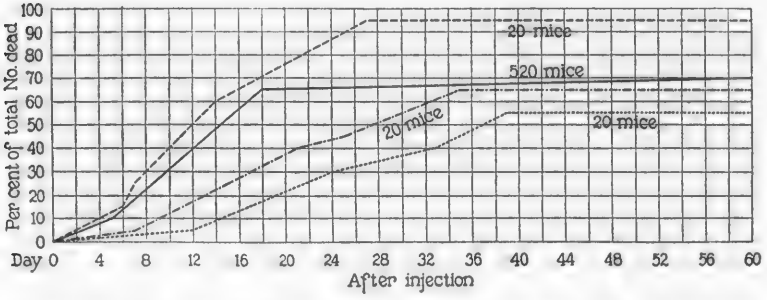
Therefore, we desired to become familiar with this curve and to determine any factors referable to host or bacteria which might alter its characteristics. By varying the host and keeping the microbic potentialities constant and later by varying the microbe and keeping

the host potentialities constant, the two factors responsible for these equilibrium curves were analyzed.

A standard host factor was arbitrarily chosen as being at least 10 mice from The Rockefeller Institute breeding room, of the same age, weighing 16 to 18 gm. each, fed on bread and milk, and living in separate glass battery jars. These mice at no time during this series of experiments showed bacilli of the paratyphoid-enteritidis group in their feces, nor did they show blood agglutinins for this group in a dilution of 1:20 to 1:80. A standard microbe factor was arbitrarily chosen as being a 16 to 24 hour heart's blood broth culture taken from the first mouse to die in any routine series such as described above. All mice and all infecting cultures, unless otherwise stated, conformed to the above specifications.

Host Factor Standardized, Microbe Factor Standardized.

It was hoped, of course, under the standard experimental conditions described above, that the mortality or equilibrium curves would



TEXT-FIG. 2. Variation in the mortality curves with the host and microbe factors standardized.

continue to be similar from series to series. And this has been true in ten series totalling 520 control mice. However, during June and July, two series of 20 mice each followed a more gradual curve and one series of 20 mice followed a more abrupt curve (Text-fig. 2). A possible explanation for these fluctuations will be discussed later, but since 90 per cent of the mice followed a similar death curve over

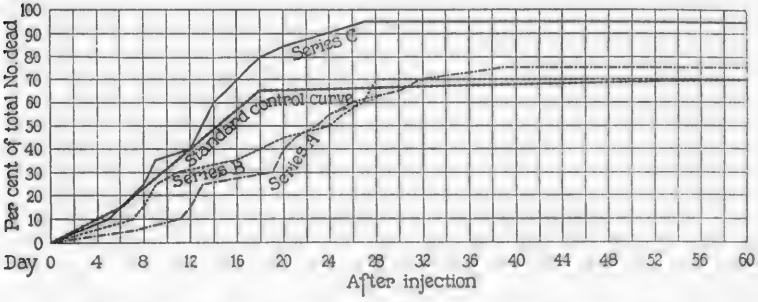
a period of 8 months and since there has been no persistent tendency to vary, the solid curve in Text-fig. 2 has been taken for a normal control.

Host Factor Varied, Microbe Factor Standardized.

The striking variation in susceptibility of mice to mouse typhoid infection has been indicated in the general work on epizootics.[5] Text-fig. 2 in this paper shows that about 20 to 30 per cent of the mice in any control series are refractory to infection *per os* and Experiment 1 reveals that these survivors may show no evidence of disease or may recover from the infection and develop agglutinins of high titer. This refractory state does not seem to be related to the normal intestinal flora;[6] it can, however, be somewhat modified by injuring the intestinal wall with ox bile.[7]

Experiment 3 was devised to bring out any possible variation in the mortality curve due to differences in age and weight of the mice employed.

Experiment 3.—20 mice, 6 weeks old, weighing 10 gm. each, called Series A, 20 mice, 1½ years old, weighing 27 gm. each, called Series B, and 20 mice, 12 weeks old, weighing 18 gm. each, called Series C, were placed in separate jars. They were all injected *per os* with an 18 hour heart's blood broth culture diluted 1:200 from the first mouse to die in a previous current series of 20. Blood cultures were taken at irregular intervals; agglutination tests were done after 6 weeks; the condition of the animals was noted; duration of life was recorded; and fatal cases were autopsied (Table II).



TEXT-FIG. 3. Mortality curves of the mice used in Experiment 3.

[6] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 21.
[7] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 33.

TABLE II.

Protocols of the Mice Used in Experiment 3.

June 22, 1922. Bacilli ingested.

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.	Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	June 23.	June 26.	June 30.	July 8.	July 18.	Aug. 14.	Aug. 14.		June 23.	June 26.	June 30.	July 8.	July 18.	Aug. 14.	Aug. 14.
A1	0	0	0	0	0	1:20 —	0	B13	0	0	0	0	D.*† July 10.		
A2	0	0	+ (1 col.).	+	D.*† July 12.			B14	0	0	0	0	0	1:20 —	0
A3	0	0	+ (1 ").	0	D.*† July 12.			B15	0	0	0	D.*† July 8.			
A4	0	+	++	D.*† July 5.				B16	0	0	0	+	0	D.*† July 20.	
A5	0	0	++	D.*† July 3.				B17	0	0	+++	D.*† July 1.			
A6	0	0	0	0	0	1:40 —	0	B18	0	0	+	D.*† July 1.			
A7	0	0	0	0	0	1:20 —	0								
A8	0	0	++	+	D.*† July 11.			B19	0	+	D.*† June 29.				
A9	0	0	0	0	0	1:20 to 1:320 ++	0	B20	0	0	0	+ (1 col.).	D.*† July 17.		
						1:640—		C1	0	0	+ (1 col.).	+	++	D.*† July 19.	
A10	0	0	0	+ (1 col.).	0	D.*† July 31.		C2	0	0	+	D.*† July 3.			
A11	0	0	+ (1 col.).	+	0	1:20—	0								
A12	0	0	0	0	0	D.*† July 24.		C3	0	0	+	++	D.*† July 9.		
A13	0	0	0	+	D.*† July 13.			C4	0	0	0	++	D.*† July 10.		
A14	0	0	0	0	0	D.*† July 22.									

A15	0	+	++	D.*† July 3.				C5	0	0	+	D.*† July 6.			
A16	0	0	0	+	D.*† July 15.			C6	0	0	+	D.*† July 5.			
A17	0	0	+	0	D.*† July 18.			C7	0	0	+	0	D.*† July 12.		
A18	0	++	D.*† June 29.					C8	0	0	D.*† June 30.				
A19	0	0	+	D.*† July 5.				C9	0	0	+	D.*† July 9.			
A20	0	0	+	0	D.*† July 16.			C10	0	0	+++	D.*† July 1.			
B1	0	0	0	0	+	D.*† July 20.		C11	0	0	0	+	++	D.*† July 19.	
B2	0	0	+	D.*† July 3.				C12	0	+ (1 col.)	D.*† July 1.				
B3	0	0	0	0	0	1:20—	0	C13	0	0	+	D.*† July 5.			
B4	0	0	+ (1 col.).	0	0	1:20—	0								
B5	0	0	++	+	++	D.*† July 19.		C14	0	++	D.*† June 28.				
B6	0	+	+ (1 col.).	0	0	1:20—	0	C15	0	0	D.*† June 30.				
B7	0	0	0	0	D.*† July 13.			C16	0	+	D.*† June 28.				
B8	0	+	D.*† June 30.					C17	0	0	0	0	0	1:20 —	0
B9	0	0	0	0	0	1:20—	0	C18	0	0	+	D.*† July 7.			
B10	0	0	0	+	D.*† July 16.			C19	0	+++	D.*† June 28.				
B11	0	0	+ (1 col.).	+	+ (1 col.).	1:20—	0	C20	0	0	+ (1 col.).	D.*† July 6.			
B12	0	D.*† June 25.													

* Autopsy lesions typical of mouse typhoid. † Heart's blood culture positive.

TABLE III.

Protocols of the Mice Used in Experiment 4.

June 22, 1922. Bacilli ingested.

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	June 23.	June 26.	June 30.	July 8.	July 18.	Aug. 14.	Aug. 14.
Penn. 1	0	0	0	0	0	D.*† July 26.	
" 2	0	0	0	0	0	1:20 —	0
" 3	0	0	0	0	0	1:20 —	0
" 4	0	0	0	0	0	1:20 —	0
" 5	0	0	0	0	0	1:20 —	0
" 6	0	0	0	0	0	1:20 —	0
" 7	0	0	0	0	0	1:20 —	0
" 8	0	0	0	0	0	1:20 —	0
" 9	0	0	0	0	0	1:20 —	0
" 10	0	0	0	+ (1 col.).	0	1:20 —	0
" 11	0	0	0	0	0	1:20 —	0
" 12	0	0	0	0	0	D.‡ July 21.	
" 13	0	0	0	0	0	1:20 —	0
" 14	0	0	0	0	D.‡ July 9.		
" 15	0	0	0	0	D.*† July 16.		
" 16	0	0	0	+	D.*† July 18.		
N. J. 6	0	0	+ (1 col.).	+ (1 col.).	D.*† July 13.		
" " 7	0	0	+ (1 ").	+ (1 ").	0	1:20 to 1:640++ 1:1,280—	0
" " 8	0	0	++	D.*† July 1.			
" " 9	0	0	++	D.*† July 5.			
" " 10	0	+	D.*† June 29.				
" " 11	0	+ (1 col.).	++	+ (1 col.).	0	1:20 —	0
" " 12	0	+	++	D.*† July 1.			
" " 13	0	0	+	D.*† July 7.			
" " 14	0	0	+	0	0	1:20 —	0
" " 15	0	0	0	+	D.*† July 13.		
" " 16	0	0	+	++	D.*† July 10.		

Penn.	17	0	0	0	0	0	1:20 —	0	N. J.	17	0	0	+	+++	D.*† July 10.		
"	18	0	0	0	0	0	1:20 —	0									
"	19	0	0	0	0	0	1:20 —	0	" "	18	0	0	+ (1 col.).	0	0	1:20 —	0
"	20	0	0	0	0	0	1:20 —	0	" "	19	0	0	+	0	D.*† July 15.		
"	21	0	0	0	0	0	1:20 —	0									
"	22	0	0	0	0	0	D.‡ July 21.		" "	20	0	0	D.*† June 29.				
"	23	0	0	0	0	0	1:20 —	0	" "	21	0	0	+ (1 col.).	0	0	D.*† July 24.	
"	24	0	0	0	0	0	1:20 —	0									
"	25	0	0	0	0	0	D.*† July 22.		" "	22	0	0	D.*† June 30.				
"	26	0	0	0	0	0	D.‡ Aug. 1.		" "	23	0	0	+ (1 col.).	+ (1 col.).	0	D.*† July 28.	
N. J.	1	0	0	+	+	D.*† July 17.			" "	24	0	+ (1 col.).	+	D.*† July 5.			
" "	2	+	0	0	D.*† July 8.				" "	25	0	0	+	D.*† July 3.			
" "	3	0	0	+ (1 col.).	0	0	1:20 —	0	" "	26	0	+	+	D.*† July 6.			
" "	4	0	0	0	0	0	1:20 —	0									
" "	5	0	+ (1 col.).	+	D.*† July 9.				" "	27	0	0	0	0	0	1:20 —	0
									" "	28	0	0	+	+	0	1:20 —	0

* Autopsy lesions typical of mouse typhoid. † Heart's blood culture positive. ‡ No lesions. Heart's blood negative.

From Text-fig. 3 it may be seen that no series was similar to the standard control curve adopted from ten series totalling 520 young adult mice. Series C, which should correspond to the standard control curve, showed an unexplainable abrupt rise with a decided increase in normal mortality rate, while Series A and B, following the standard curve more closely, showed the flattening encountered during the early summer experiments. An explanation for these fluctuations will be discussed later. The curves, however, do present evidence that young, adult, and old mice of these experiments show no striking difference in susceptibility to mouse typhoid infection.

From Table II it may be seen that most of the survivors in Series A, B, and C showed no positive blood cultures, no agglutinins, and remained well throughout the experiment. Mouse A 9, although blood cultures were negative, showed agglutinins in low titer; Mice A 11, B 4, B 6, and B 11 showed positive blood cultures but no agglutinins.

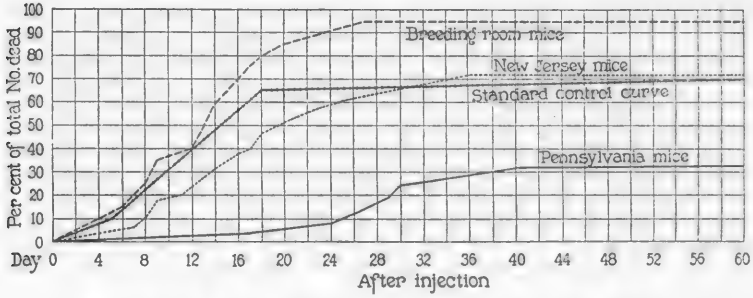
Experiment 4 was designed to bring out any possible variation in the mortality curve due to differences in breed of mice.

Experiment 4.—26 mice from a Pennsylvania breeder and 28 mice from a New Jersey breeder were placed in separate jars. Stool cultures, examined twice, were negative; blood cultures were negative; and agglutination tests were negative in dilutions of 1:20 and 1:40. The injection was carried out simultaneously with Experiment 3, consequently the same bacterial suspension and same controls were used. Subsequent procedures were similar to those employed in Experiment 3.

Text-fig. 4 shows that different breeds of mice vary in their susceptibility to mouse typhoid infection. While the New Jersey mice followed a curve quite similar to that of the 520 standard controls, the Pennsylvania mice were more refractory to infection.

Table III analyzes this difference. While nearly all the survivors in the Pennsylvania series showed negative blood cultures and agglutinins throughout the experiment, the New Jersey mice were less resistant. Not only did a much higher per cent succumb, but the survivors followed a different course. Nos. 4 and 27 were entirely refractory; Nos. 3, 11, 14, 18, and 28 showed positive blood cultures, but no agglutinins; No. 7 showed positive blood cultures and homologous agglutinins.

In brief, then, observation and experiment thus far have shown that 20 to 30 per cent of any series of mice taken from The Rockefeller Institute breeding room are not susceptible to mouse typhoid infection from a certain strain of *Bacillus pestis caviæ*. These mice may discharge the bacilli for a few days with the feces, but at no time are blood cultures positive nor are agglutinins demonstrable. An additional 5 or 10 per cent of the mice show symptoms of disease and positive blood cultures but survive and may later develop agglutinins. The remaining, approximately 70 per cent, pass the ingested bacilli *per rectum*, show positive blood cultures, and die in a more or less constant ratio, relative to time. Age appears not to affect susceptibility; source of stock or breed, on the other hand, is an important factor.



TEXT-FIG. 4. Mortality curves of the mice used in Experiment 4.

In general, susceptibility must be considered a relative and graded property, for although individuals appear to fall into rough groups, a study of many protocols will show occasional intermediate and varied responses to the bacteria by the host.

The agglutination phenomenon is no criterion of immunity; a surviving mouse may or may not give a positive reaction.

Microbe Factor Varied; Host Factor Standardized.

After becoming familiar with the general range of host variability, it was desired to vary the microbe factor and to determine its effect upon the mortality curves. The effect of dosage variation was first tried.

TABLE IV.

Protocols of the Mice Used in Experiment 5.

June 7, 1922. Bacilli ingested.

Mouse No.	Blood cultures.								Agglutinins.	Blood culture.
	June 8.	June 9.	June 10.	June 12.	June 17.	June 21.	June 27.	July 5.	July 18.	July 20.
Series 1.										
1, male.	+ (1 col.).	+ (1 col.).	+ (1 col.).	+	+	+	D.*† June 23.			
2, "	0	0	0	+	0	0	D.*† June 23.			
3, "	0	0	+	++	++	+	D.*† June 22.			
4, "	0	0	0	+ (1 col.).	0	0	0	0	D.*† July 15.	
5, "	0	0	0	++	D.*† June 18.					
6, "	0	0	0	0	+	0	0	+ (1 col.).	D.*† July 13.	
7, "	0	0	0	+	+	D.*† June 21.				
8, "	0	0	0	0	0	0	0	0	1:20 to 1:1,280 ++ 1:2,560 −	0
9, "	0	0	0	+	++	D.*† June 19.				
10, "	0	0	0	0	0	D.*† June 22.				
11, female.	0	0	0	0	0	0	0	0	1:20 −	0
12, "	0	0	0	0	++	D.*† June 19.				

13, female.	0	0	0	+	D.*† June 18.					
14, "	0	0	0	+	0	0	0	0	1:20 −	0
15, "	0	0	0	0	0	+	0	D.*† July 5.		
16, "	0	0	0	0	0	0	0	D.*† June 30.		
17, "	0	0	0	+	0	0	0	0	1:20 to 1:320 ++ 1:640 −	0
18, "	+ (1 col.).	++	+++	D.*† June 12.						
19, "	0	+	+++	D.*† June 12.						
20, "	0	0	0	0	0	0	0	0	1:20 to 1:80 ++ 1:160 −	0
Series 2.										
1, male.	0	0	0	0	+	++	D.*† June 23.			
2. "	0	0	0	+	+	+	+	++	D.*† July 10.	
3, "	0	0	0	+ (1 col.).	0	0	D.*† June 28.			
4, "	0	0	0	0	+ (1 col.).	+ (1 col.).	0	0	D.*† July 11.	
5, "	0	0	0	0	D.*† June 16.					
6, "	0	0	0	0	+ (1 col.).	+ (1 col.).	0	D.*† July 1.		

* Autopsy lesions typical of mouse typhoid. † Heart's blood culture positive.

TABLE IV—*Continued*.

Mouse No.	Blood cultures								Agglutinins.	Blood culture.
	June 8.	June 9.	June 10.	June 12.	June 17.	June 21.	June 27.	July 5.	July 18.	July 20.
7, male.	0	0	0	0	++	++	D.*† June 27.			
8, "	0	+	+	0	0	0	0	0	1:20 —	0
9, "	0	0	0	0	0	0	0	0	D.* July 10.	
10, "	0	0	0	0	0	0	0	0	1:20 —	0
11, female.	0	0	0	+	++	++	+ (1 col.).	+	D.*† July 13.	
12, "	0	0	+ (1 col.).	0	0	0	0	0	D. July 18.	
13, "	0	0	0	0	0	0	0	0	1:20 —	0
14, "	+ (1 col.).	0	0	++	++	D.*† June 18.				
15, "	0	0	0	+	+	+	D.*† June 24.			
16, "	0	0	0	0	0	0	D.*† June 24.			
17, "	0	0	0	0	+	+	0	D.*† July 3.		
18, "	0	0	0	0	+	++	D.*† June 28.			
19,	0	0	0	0	++	D.*† June 19.				
20, "	0	0	0	+ (1 col.).	0	0	0	+ (1 col.)	D.*† July 7.	

Series 3.										
1, male.	0	0	0	0	0	0	0	0	1:20 —	0
2, "	0	0	0	0	0	0	0	0	1:20 —	0
3, "	0	0	0	0	+ (1 col.).	+	D.*† June 28.			
4, "	0	0	0	0	0	0	0	0	1:20 —	0
5, "	0	0	0	0	0	0	0	0	1:20 —	0
6, "	0	0	0	+ (1 col.).	+ (1 col.).	+	+	++	D.*† July 8.	
7, "	0	+	+	++	D.*† June 15.					
8, "	0	0	0	0	0	++	D.*† June 24.			
9, "	0	0	0	0	0	0	0	0	1:20 —	0
10, "	0	0	0	+ (1 col.).	+ (1 col.).	0	0	0	D.*† July 6.	
11, female.	0	0	0	0	++	++	D.*† June 26.			
12, "	0	0	0	0	0	+ (1 col.).	+	D.*† July 3.		
13, "	0	0	0	0	++	D.*† June 19.				
14, "	0	0	0	0	++	D.*† June 19.				
15, "	0	0	0	0	++	D.*† June 19.				
16, "	0	0	0	0	0	0	0	0	D.*† July 12.	
17, "	0	0	0	0	0	0	+	D.*† July 2.		

TABLE IV—*Continued.*

Mouse No.	Blood cultures.								Agglutinins.	Blood culture.
	June 8.	June 9.	June 10.	June 12.	June 17.	June 21.	June 27.	July 5.	July 18.	July 20.
18, female.	0	0	+ (1 col.).	++	D.*† June 14.					
19, "	0	0	0	0	0	0	0	0	1:20—	0
20, "	0	0	0	0	0	0	0	0	1:20 to 1:5,120++ 1:10,240—	0
Series 4.										
1, male.	0	0	0	++	D.*† June 15.					
2, "	0	0	0	0	0	0	0	0	1:20—	0
3, "	0	0	0	0	+	+	0	0	1:20 to 1:80 ++ 1:160—	D.*† July 24.
4, "	0	0	0	0	0	0	+	0	1:20—	0
5, "	0	0	0	0	0	0	0	0	1:20—	0
6, "	0	0	0	0	0	0	0	0	D.*† July 19.	
7, "	0	0	0	0	D.*† June 17.					
8, "	0	0	0	0	0	0	0	0	1:20 to 1:80 ++ 1:160—	0
9, "	0	0	0	++	D.*† June 15.					
10, "	0	0	0	0	+	+	+	D.*† July 4.		
11, female.	0	0	0	0	0	0	0	0	1:20—	0

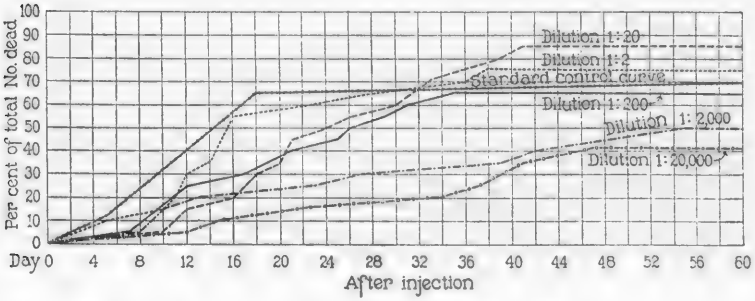
12, female.	0	0	0	0	0	0	0	0	D.*† July 16.	
13, "	0	0	0	0	0	0	0	0	1:20—	0
14, "	0	0	0	0	0	0	0	0	1:20—	0
15, "	0	0	0	0	0	0	0	0	1:20—	0
16, "	0	0	0	0	0	0	0	0	1:20—	D. Aug. 2.
17, "	0	0	0	0	0	D.*† June 20.				
18, "	0	0	0	0	0	0	0	0	1:20—	0
19, "	0	0	0	0	0	0	0	0	1:20—	0
20, "	0	0	0	0	+ (1 col.).	+	0	D.*† June 30.		
Series 5.										
1, male.	0	0	0	+ (1 col.).	+	D.*† June 22.				
2, "	0	0	0	0	0	0	0	0	1:20—	0
3, "	0	0	0	0	0	0	0	0	D.*† July 11.	
4, "	0	0	0	0	0	0	0	0	1:20—	0
5, "	0	0	0	0	0	0	0	D. June 29.		
6, "	0	0	0	0	0	0	0	0	1:20—	0
7, "	0	0	0	+ (1 col.).	+	+	+	0	D.*† July 14.	
8, "	0	0	0	0	0	0	0	0	1:20—	0
9, "	0	0	0	0	0	0	0	0	D. July 18.	
10, "	0	0	0	0	0	0	0	0	1:20—	0

TABLE IV—*Concluded.*

Mouse No.	Blood cultures.								Agglutinins.	Blood culture.
	June 8.	June 9.	June 10.	June 12.	June 17.	June 21.	June 27.	July 5.	July 18.	July 20.
11, female.	0	0	0	0	0	0	0	0	1:20—	0
12, "	0	0	0	0	++	D.*† June 19.				
13, "	0	0	0	0	0	0	0	0	1:20—	0
14, "	0	0	0	0	0	0	0	0	1:20—	0
15, "	0	0	0	0	0	+	0	0	1:100 to 1:3,200 ++ 1:6,400—	0
16, "	0	0	0	0	0	0	0	0	1:20—	D.*† July 24.
17, "	0	0	0	0	0	0	0	0	1:20—	0
18, "	0	0	0	0	0	0	0	0	D.*† July 18.	
19, "	0	0	0	0	0	0	0	0	1:20—	0
20, "	0	0	0	0	0	0	0	0	1:20—	0

Experiment 5.—100 mice from the breeding room, weighing 16 to 18 gm. each, were placed in separate jars. An 18 hour heart's blood broth culture from the first mouse to die of a previous current series was used for the injections. It was completely agglutinated in its homologous serum diluted 1:10,000; intraperitoneally it killed mice in 5 days at a dilution of 1:10,000; there were approximately 1 billion organisms per cc. in the broth culture.

Series 1, 10 males and 10 females, was given *per os* a dilution of 1:2 (0.5 cc. of the undiluted broth culture); Series 2, 10 of each sex, received a dilution of 1:20; Series 3, a dilution of 1:200; Series 4, a dilution of 1:2,000; and Series 5, a dilution of 1:20,000. Blood cultures were taken at short intervals; the condition of the animals was noted; agglutination tests were done after 5 weeks; duration of life was charted; and autopsies were performed on fatal cases.



TEXT-FIG. 5. Mortality curves of the mice used in Experiment 5.

Text-fig. 5 presents the results of this experiment in a striking manner. The curves of Series 1, 2, and 3 are grouped about the standard control curve and, except for a flattening, are roughly similar to it and to each other. The curves of Series 4 and 5, however, similar to each other, have a very gradual slope. It seems, therefore, that the massive lethal dose of a 1:200 dilution or less selects a relatively constant number of susceptibles, roughly 70 to 80 per cent of the total number used. And a dilution of 1:2,000 to 1:20,000 selects about 40 to 50 per cent. These observations, together with the results of Experiment 1, would indicate that the susceptibility of a population to mouse typhoid is a relative and graded property. Just how enduring this quality may be has not been determined, but so far it is clear that regardless of dosage 20 to 30 per cent of these mice are immune to *Bacillus pestis caviæ* infection *per os*.

TABLE V.

Agglutination Titers of the Cultures Used in Experiment 6.

Culture No.	Serum dilution.											
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	1:40,960
1	++	++	++	++	++	++	++	++	++	++	+1	−
2	++	++	++	++	++	++	++	++	++	++	−	−
3	++	++	++	++	++	++	++	++	++	++	+1	−
4	++	++	++	++	++	++	++	++	++	++	−	−
5	++	++	++	++	++	++	++	++	++	++	+1	−

++ indicates complete agglutination; +1, marked agglutination, supernatant fluid not clear; −, no agglutination.

From Table IV it is seen that while the survivors of Series 2, 3, 4, and 5 fall regularly into the groups indicated above, those surviving the largest dose usually showed positive blood cultures and agglutinins.

Experiment 6 was planned to compare the infectivity of the current strain with carrier cultures, chronic septicemia cultures, and a laboratory stock culture which had not had an animal passage for 2½ years.

Experiment 6.—100 mice from the breeding room, weighing 16 to 18 gm. each, were placed in separate jars. 20 were injected *per os* by stomach tube with Culture 1, 4 billion organisms per cc., diluted 1:200; 20 were similarly injected with Culture 2, 3 billion organisms per cc.; 20 with Culture 3, 2 billion organisms per cc.; 20 with Culture 4, 1 billion organisms per cc.; and 20 with Culture 5, 8 billion

TABLE VI.

Intraperitoneal Titers of the Cultures Used in Experiment 6.

Culture No.	Broth dilution.				
	1:20	1:200	1:2,000	1:20,000	1:200,000
	days	*days*	*days*	*days*	*days*
1	1, 1	1, 3	3, 4	5, 40+	4, 8
2	1, 1	1, 3	3, 7	3, 7	5, 7
3	1, 1	1, 1	4, 4	3, 6	4, 40+
4	1, 1	3, 4	5, 5	4, 8	5, 8
5	1, 1	2, 3	6, 6	7, 8	7, 20

Two mice were injected for each dilution. The figures refer to the duration of life after injection. Survivors were discarded after 40 days.

organisms per cc. Agglutinations were done on the five cultures (Table V); a numerical estimate was made of the number of organisms per cubic centimeter in the 18 hour broth cultures; and intraperitoneal titrations were done (Table VI). The animals injected *per os* were observed daily. Blood cultures were taken; duration of life was recorded; and autopsies were performed on the fatal cases (Table VII).

Culture 1 was an 18 hour heart's blood broth culture from the first animal to die in a previous routine series of 20 mice. Culture 2 was an 18 hour tail blood broth culture from a mouse which had been injected *per os* 5 weeks previously. Cultures 3 and 4 were obtained from the stools of two separate mice which had been infected 5 weeks previously. Culture 5 was an 18 hour broth culture of the stock original strain which had not had an animal passage on artificial media for 2½ years.

TABLE VII.

Protocols of the Mice Used in Experiment 6.

June 30, 1922. Bacilli ingested.

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	July 1.	July 5.	July 7.	July 10.	July 17.	Aug. 15.	Aug. 15.
Series 1.							
1	+	+	+++	D.*† July 8.			
2	0	0	+ (1 col.).	+	D.*† July 16.		
3	0	++	+++	D.*† July 8.			
4	0	0	+	++	D.*† July 17.		
5	0	0	+	+	+	D.*† July 26.	
6	0	+ (1 col.).	0	+	D.*† July 13.		
7	0	++	D.*† July 7.				
8	0	0	0	+	0	1:20 —	0
9	0	0	0	0	0	1:20 —	0
10	0	++	++	++	D.*† July 11.		
11	0	+	++	D.*† July 10.			
12	0	+ (1 col.).	+	++	D.*† July 13.		

13	0	0	0	0	0	1:20 −	0
14	0	++	++	D.*† July 9.			
15	0	0	+	+	+	D.*† July 28.	
16	0	+ (1 col.).	+	0	0	1:20 −	0
17	0	0	+	+	D.*† July 17.		
18	0	0	0	0	0	1:20 −	0
19	0	0	+ (1 col.).	+	+ (1 col.).	1:20 −	0
20	0	+	++	++	D.*† July 13.		
Series 2.							
1	0	0	+ (1 col.).	+	D.*† July 14.		
2	0	0	+	+	D.*† July 13.		
3	0	0	+	D.*† July 10.			
4	0	+	+	+	+	1:100 to 1:400 ++ 1:800 −	0
5	0	+	+	+	D.*† July 15.		
6	0	0	+ (1 col.).	0	0	1:20 −	0
7	0	0	+ (1 ").	0	0	1:20 to 1:320 + 1:640 −	0
8	0	+++	D.*† July 6.				

* Autopsy lesions typical of mouse typhoid. † Heart's blood culture positive.

TABLE VII—*Continued.*

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	July 1.	July 5.	July 7.	July 10.	July 17.	Aug. 15.	Aug. 15.
9	0	++	D.*† July 7.				
10	0	0	+	+	D.*† July 13.		
11	0	0	0	+ (1 col.).	D.*† July 16.		
12	0	++	D.*† July 7.				
13	0	0	+ (1 col.).	0	0	1:20 to 1:640 ++ 1:1,280 –	0
14	0	0	+	+	0	1:20 –	0
15	0	+	D.*† July 6.				
16	+	0	+	+	0	1:20 to 1:1,280 ++ 1:2,560 –	0
17	0	0	0	0	0	D.*† Aug. 10.	
18	0	0	+	+ (1 col.).	+	1:20 –	0
19	0	0	+ (1 col.).	+	+	1:20 –	0
20	0	0	+ (1 ").	+	0	D.*† Aug. 7.	
Series 3.							
1	0	0	+ (1 ").	0	0	1:20 –	0
2	0	+	0	+	0	1:20 –	0

3	0	++	+++	D.*† July 8.			
4	0	++	D.*† July 8.				
5	0	0	+	D. *† July 10.			
6	0	+++	D.*† July 6.				
7	0	0	+ (1 col.).	0	+	D.*† July 28.	
8	0	D.*† July 6.					
9	0	0	+ (1 col.).	+	+	D.*† July 20.	
10	0	+	+	+++	D.*† July 13.		
11	0	0	0	0	0	1:20 —	0
12	0	+	0	+	+	1:20 —	0
13	0	+	++	D.*† July 10.			
14	0	+	D.*† July 7.				
15	0	+	+	0	D.*† July 13.		
16	0	++	+++	D.*† July 8.			
17	0	+ (1 col.).	0	0	0	D.*† July 24.	
18	0	0	0	0	0	1:20 —	0
19	0	0	+ (1 col.).	0	0	1:20 —	0
20	0	0	0	0	0	1:20 —	0

TABLE VII—*Continued.*

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	July 1.	July 5.	July 7.	July 10.	July 17.	Aug. 15.	Aug. 15.
Series 4.							
1	0	+	+	+	D.*† July 12.		
2	+	++	++	D.*† July 9.			
3	+ (1 col.).	+	D.*† July 10.				
4	0	0	0	+	+	D. *† July 24.	
5	0	0	0	0	0	1:20 —	0
6	0	0	+	++	D.*† July 14.		
7	0	0	+	+ (1 col.).	0	1:20 —	0
8	0	+ (1 col.).	+	D.*† July 10.			
9	0	0	+ (1 col.).	+ (1 col.).	0	1:20 —	0
10	0	0	+	+	+ (1 col.).	1:20 to 1:80 ++ 1:160 —	0
11	0	++	D.*† July 7.				
12	0	0	0	+	0	D.*† July 24.	
13	0	+ (1 col.).	+ (1 col.).	+	D.*† July 15.		
14	0	+	+++	D.*† July 8.			

15	0	+	+	++	D.*† July 14.		
16	0	0	0	+	D. * † July 16.		
17	0	0	0	+	0	1:20 –	0
18	0	+ (1 col.).	0	+	+	D.*† July 22.	
19	0	0	0	0	0	1:20 –	0
20	0	0	++	D.*† July 9.			
Series 5.							
1	0	0	0	0	0	1:20 –	0
2	0	+	+++	D.*† July 8.			
3	0	0	0	0	0	1:20 –	0
4	0	0	+	+	D.*† July 17.		
5	0	0	0	0	0	1:20 –	0
6	0	+	+++	D.*† July 11.			
7	0	0	0	+ (1 col.).	0	1:20 –	0
8	0	0	0	+ (1 ").	0	1:20 –	0
9	0	0	0	0	0	1:20 –	0
10	0	0	+	D.*† July 9.			
11	0	0	+	D.*† July 10.			
12	0	0	0	0	0	1:20 –	0
13	0	+ (1 col.).	+	+	D.*† July 13.		

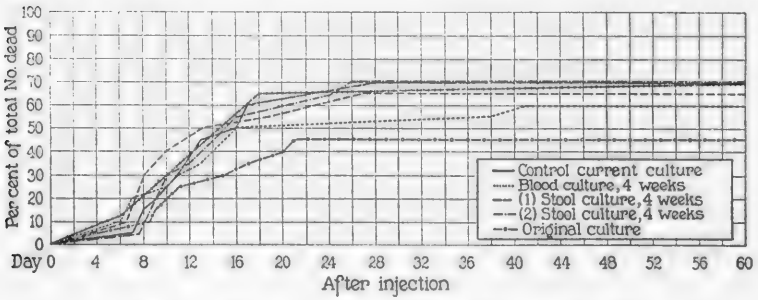
TABLE VII—*Concluded.*

Mouse No.	Blood cultures.					Agglutinins.	Blood culture.
	July 1.	July 5.	July 7.	July 10.	July 17.	Aug. 15.	Aug. 15.
14	0	+	++	D.*† July 8.			
15	0	+ (1 col.).	+ (1 col.).	0	0	1:20 –	0
16	0	+ (1 ").	+	+	+	D.*† July 20.	
17	0	0	0	0	0	1:20 –	0
18	0	0	0	+	0	1:20 –	0
19	0	+ (1 col.).	+	+	D.*† July 15.		
20	0	0	+	+	+ (1 col.).	D.*† July 21.	

The mortality curves of the current culture and the two stool carrier cultures follow the standard control curve very closely (Text-fig. 6). The series injected with the chronic septicemia culture and the series injected with the original stock strain show somewhat more gradual slopes.

The agglutination titer and intraperitoneal titer of all five series were about the same (Tables V and VI).

Table VII analyzes the host reaction to the various strains. The survivors of Series 1, 3, 4, and 5 showed for the most part no positive blood cultures and no agglutinins; the survivors of Series 2, however, usually showed positive blood cultures and agglutinins.

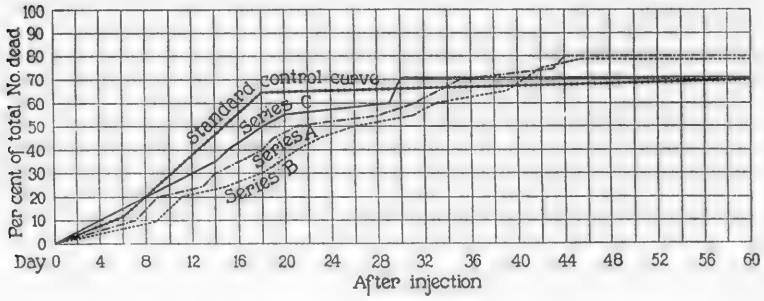


TEXT-FIG. 6. Mortality curves of the mice used in Experiment 6.

Experiment 7 was planned to compare the infectivity of a culture taken from an animal early in the disease with cultures taken shortly after death and 6 days after death.

Experiment 7.—A series of 10 mice was infected as usual with an 18 hour heart's blood broth culture from the first mouse to die of a preceding series. 4 days later, blood cultures on 6 of these mice were positive and a culture from 1 of the mice (No. 8) was used to infect 20 mice *per os* in a dilution of 1:200, 3 billion colonies per cc., and 8 mice intraperitoneally in dilutions of 1:100, 1:1,000, 1:10,000, and 1:100,000. 3 days later, or 7 days after injection, the original mouse (No. 8) died and was again cultured. The resulting culture, 10 billion colonies per cc., was injected into a new series precisely as above. The dead mouse was then kept aerobically at room temperature for 6 days and cultured again. The resulting culture, 1 billion per cc., was injected as above.

From Text-fig. 7 it may be seen that the mortality curves of these series vary but little from the standard control curve. Also the intraperitoneal virulence titration was similar in the three series.



TEXT-FIG. 7. Mortality curves of the mice used in Experiment 7.

DISCUSSION.

The concept of variation in host susceptibility to infection has had need of experimental demonstration and measurement.

Fluctuation of microbic virulence, on the other hand, has engaged the attention of bacteriologists and immunologists for the past decade and has occasioned an enormous amount of experimental study. Such investigation with microbes gaining entrance by an abnormal portal of entry to a foreign host or growing on artificial media results in considerable variation, but the few experiments detailed above, in which bacteria were used which gain entrance to their native host by the normal portal of entry, elicit so far, under natural conditions, but little evidence for change in bacterial virulence.

Concerning the unexplained fluctuations in the standard control curves, this much may be said. During the summer a small outbreak of mouse typhoid occurred among the normal breeding stock. And it was at this time that the mice used in the experiments detailed above followed more acute or more gradual mortality curves. Quite possibly, the animals may have been incipient cases or may have acquired some degree of resistance which affected the death rate. Evidence for this hypothesis will be presented at another time.

CONCLUSIONS.

Mice bred at The Rockefeller Institute vary in their susceptibility to mouse typhoid infection caused by a certain strain of *Bacillus pestis caviæ*.

This graded variation may be roughly analyzed as follows: in any series infected *per os* with a fixed dose, 20 to 30 per cent show no sign of infection, no positive blood cultures, and no agglutinins; 5 or 10 per cent present symptoms of disease, positive blood cultures, and then recover with or without homologous agglutinins; 70 or 80 per cent develop positive blood cultures and succumb in a more or less constant ratio relative to time.

The strain of *Bacillus pestis caviæ* employed throughout a 10 month series of experiments has shown no permanent change in virulence. Blood cultures taken from infected mice early in disease, shortly after death, and 6 days after death, chronic stool carrier cultures, and chronic septicemia cultures, all show approximately the same degree of virulence.

CONTRIBUTION TO THE MANNER OF SPREAD OF MOUSE TYPHOID INFECTION.

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(Received for publication, August 25, 1922.)

In the preceding paper[1] we state that the spread of mouse typhoid in a given mouse population is determined by three factors, or variables; namely, the spacial and quantitative distribution of the bacilli, the virulence of the microorganism, and the susceptibility of the host. That the last two factors are concerned in this process has been shown by the experiments in which the bacilli remained virtually of constant virulence while the susceptibility of the mice varied considerably. In the present paper we propose to describe experiments dealing more specifically with the factor of bacillary distribution under circumstances in which the mice are exposed to infection through the normal channels and without artificial instrumentation. Moreover, using the experimental results recorded in this paper and the preceding one as a basis, we have compared our standard control curve[1] with the epidemic curve as given by Amoss[2] for mouse typhoid epizootics and, having discovered a striking correspondence between them, have suggested an explanation of the manner in which the epidemic curve is produced.

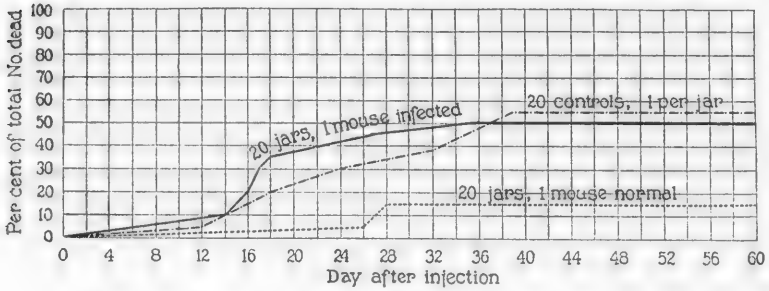
Spacial and Quantitative Distribution of Bacilli.

In the experiments already reported[1] each mouse was given a fixed and constant amount of the routine culture of mouse typhoid bacilli (*Bacillus pestis caviæ*)[3] *per os* through a specially devised tube. In the tests now to be described, contact experiments were arranged in such a manner that healthy mice and mice inoculated by means of the tube

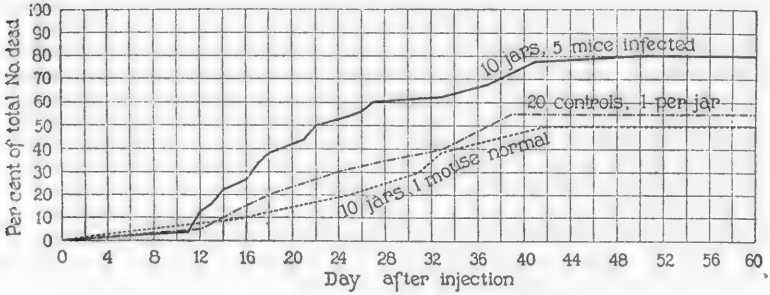
[1] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.
[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.
[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

per os were brought together in a way calculated to vary the concentration of the available infecting bacilli.

Experiment 1.—100 normal mice, bred at The Rockefeller Institute, weighing 16 to 18 gm. each, were assembled as follows: Series A consisted of 20 jars containing 2 mice each; Series B consisted of 10 jars containing 6 mice each. Of Series A, 1 mouse in each jar, and of Series B, 5 mice in each jar received by stomach tube 0.5 cc. of an 18 hour heart's blood broth culture, diluted 1:100. The activity of the culture was controlled by means of 20 mice in separate jars, each of which was given a similar dose.



TEXT-FIG. 1. Mortality curves of Series A, Experiment 1.



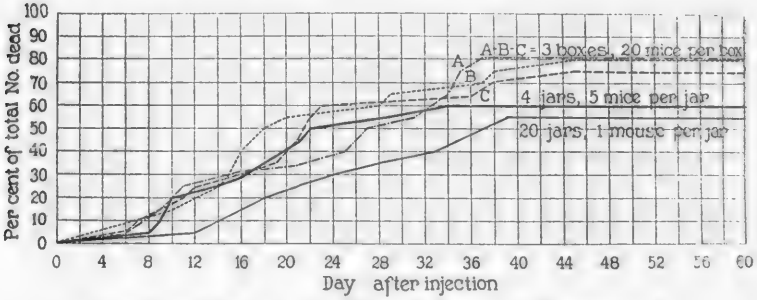
TEXT-FIG. 2. Mortality curves of Series B, Experiment 1.

In this experiment each jar of Series A contained 1 inoculated and 1 uninoculated mouse, while in Series B each jar contained 5 inoculated and 1 uninoculated mouse. It was therefore probable that the single contact mouse per jar in Series B would be exposed to and hence would ingest a larger number of bacilli voided by the 5 inoculated

mice than would the single contact mouse in Series A, in which only 1 inoculated mouse shared the jar. Text-figs. 1 and 2, which reproduce the mortality curves of the two series, exhibit the different results attained which are in conformity with the prediction.

The figures bring out the strikingly different effects induced by mere dosage of the bacilli alone. Thus in Series B, in which the ratio of inoculated to uninoculated mice was 5:1, the mortality curve of the contact mice agrees closely with that of the control curve based on the 20 mice each of which was given a fixed dose of the bacilli; while in Series A, in which the ratio was 1:1, the mortality of the contact mice is almost negligible. The results of the two tests are so impressive that they may be taken as indicating that with a homogeneous mouse stock the factor of dosage alone, conditioned only upon the quantity of bacilli available being large, is sufficient to induce massive infection leading to death, while conversely when the available dosage is small it is insufficient to produce this severe effect.

The next experiment was planned in a way to enable us to show the influence of crowding upon a series of mice all of which had been given *per os* a uniform dose of mouse typhoid bacilli.



TEXT-FIG. 3. Mortality in Cages A, B, and C, Experiment 2.

Experiment 2.—100 mice from the breeding room, weighing 16 to 18 gm. each, were assembled as follows: 20 mice were placed in each of 3 separate cages measuring 14 by 14 by 14 inches and designated A, B, and C. 5 mice were placed in each of 4 cages (battery jars) measuring 8 by 10 inches. 1 mouse was placed in each of 20 battery jars of the same dimensions as the preceding. These last single mice served also as controls for Experiment 1. Each mouse of the various series contained in the several jars received *per os* by tube 0.5 cc. of an 18 hour heart's blood broth culture, diluted 1:100. This culture was also used in Experiment 1.

Text-fig. 3 shows that the mortality in Cages A, B, and C, in which the crowding of the mice was greatest, was higher than in the jars in which the crowding was less; and the mortality was least among the isolated control mice.

The explanation of the differences is obvious; the mice in Cages A, B, and C received not only a fixed initial dose of the culture but large additional quantities eliminated by the sick mice with the dejecta and through the devoured dead companions. In this way even the more resistant mice become infected and tend to succumb. From all of which it may be concluded that degree of distribution or dosage of the bacilli is a determining factor in the increased death rate.

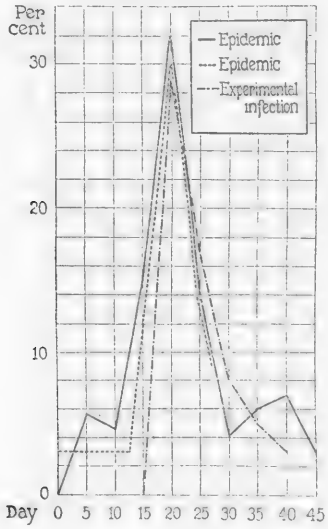
Comparison of Standard Control and Experimental Epidemic Curves.

Experiments, carried out on 10 series of mice from The Rockefeller Institute breeding room totaling 520 individuals, have shown that when a given dose of mouse typhoid bacilli is administered *per os*, the mortality curve is, generally speaking, a constant one. We refer to this curve as the standard control curve.

In order to compare the standard curve with the curve based on epidemics of mouse typhoid, we plotted the former with coordinates similar to those employed by Amoss in recording the mortality in experimentally induced mouse typhoid epidemics. The resulting curve parallels closely Amoss' experimental epidemic curves which followed the introduction of a fresh population into an infected community.[2] This parallel is shown in Text-fig. 4. Hence the question presents itself whether the factors accounting for the standard curve may not also explain the epidemic curve. Generally, of course, it is unwise to relate causally independent series of phenomena merely because they may be described by the same type of curve. In this instance, however, in which conditions are so closely allied and in which we are comparing controlled phenomena with like uncontrolled phenomena, a similar causal relation on the basis of similarity of curves seems more justifiable.

Our experiments show that the standard control curve arises from the spacial and quantitative distribution factor; that is, from the artificial injection *per os* of mouse typhoid bacilli or from conditions

described in this paper (Experiment 1). This being the case, it may be inferred that the epidemic curve is initiated by a like distribution factor acting upon a susceptible population. Furthermore, the character of this standard control curve represents an equilibrium between microbic virulence and host susceptibility, and since it is found that under the conditions of the experiments virulence remains constant while susceptibility varies, it would appear that variation in the form of this curve results from differences in susceptibility



TEXT-FIG. 4. Comparison of the standard control curve with experimental epidemic curves.

of the mice. From these considerations we have concluded that the form of the curves given by Amoss for experimentally induced epidemics of mouse typhoid depends upon the degree of susceptibility of a mouse population simultaneously exposed to a fixed and infectious dose of mouse typhoid bacilli.

In using the standard control curve to explain the mortality figures in a spontaneously occurring epidemic of mouse typhoid, we are on less sure ground since the conditions of exposure may be less constant than those existing in the experimental epidemics, whence it follows

that the curve will be irregular and rise and fall with the fluctuation of the spacial distribution of the bacilli as well as with the varying individual susceptibilities of the mice.

The influence of the distribution factor is exhibited, in our opinion, by Topley's[4] studies in which many mice were kept together in a single large cage to which fresh increments were added from time to time. We believe that the quantitative distribution factor in these experiments was affected by the care given the cage (removal of refuse, etc.), by the number and individual quality of the mice, and by the death rate. A high death rate among a recently added population indicated great concentration of the bacilli; a low death rate the reverse condition. In other words, Topley's curves can be accounted for without assuming a change in infecting power of the mouse typhoid bacilli.

Similarly the experimental results of Amoss[2] in which the mice were assembled in groups of 5 and 10 can be explained on the basis of the spacial and quantitative distribution of the bacilli alone, in the first instance by the attendant and in the next by the number of mice acquiring and succumbing to infection.

CONCLUSION.

The experiments reported in this paper and in the preceding one[1] indicate that with a given susceptible mouse population and a certain strain of mouse typhoid bacilli the sporadic and epidemic prevalences of mouse typhoid are determined by the spacial and quantitative distribution of the bacilli.

Under circumstances in which the entire mouse population is so exposed as to be in direct contact with an infecting dose of the mouse typhoid bacillus, the nature of the resulting mortality curve depends upon the quality of susceptibility of the individuals composing the population.

[4] Topley, W. W. C., *Lancet*, 1919, ii, 1; *J. Hyg.*, 1920–21, xix, 350; 1921, xx, 103. Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227. Topley, W. W. C., *J. Hyg.*, 1922, xxi, 10, 20.

FURTHER STUDIES ON BACTERIAL HYPERSUSCEPTIBILITY. II.

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Plate 14.

(Received for publication, July 29, 1922.)

The work reported in this paper is a direct continuation of studies begun several years ago and published in part in a preceding communication by one of the writers (1), in which it was shown that intradermal and presumably, also, general tuberculin reactions could be distinctly differentiated from true anaphylaxis to tuberculoprotein.

As a consequence of the work done at that time, it appeared that in the course of infections with the tubercle bacillus, two distinct varieties of hypersensitiveness may develop. One of these, true anaphylaxis against the protein materials of the bacteria, occurs late in prolonged infections, not sooner than 3 weeks after first lodgment of the living bacteria in the body. This form of hypersusceptibility is analogous to such phenomena as horse serum and other protein anaphylaxis and expresses itself in generalized hypersusceptibility, in the uterine phenomena of Dale, and, as far as the skin is concerned, in the rapid, urticaria-like, evanescent skin reactions so familiar to all physicians who have tested pneumonia patients for horse serum hypersusceptibility by intradermal injections. The other, the tuberculin reaction, occurs much earlier, appearing in tuberculous animals within 8 or 9 days after a massive injection of living bacilli, thus developing several weeks before the anaphylactic reaction appears, and is associated with a skin reaction slower in development and accompanied by deeper pathological injury and slower healing. This reaction, analogous apparently in every way to the so called typhoidin, mallein, abortin, and similar reactions, can be elicited in the infected animal by injection with a residue of tubercle bacillus extract from which all proteins have been removed as far as this can be done by the usual methods of precipitation with acid and boiling in an acid reaction. Moreover, whereas true protein anaphylaxis can be induced in the animals by repeated injections of whole dead bacteria or bacterial extracts, the latter type, or the tuberculin type of reaction, occurs only in animals actually infected with the living organisms, an important point previously observed by Römer (2), Baldwin (3), Krause (4), and others.

We thus have in addition to, and distinct from true protein anaphylaxis, an apparently specific hypersensitiveness associated only with infection, and induced in the sensitive animal with derivatives of the bacteria the chemical structure of which is uncertain but which certainly differ materially from the substances ordinarily spoken of as proteins. Our suspicion that we are dealing with substances of lower molecular structure than proteins is now being investigated by chemical analysis and will be reported in a subsequent paper. Until this work is finished, however, no statement concerning the exact chemical nature of these materials is justified. For possible explanations of the particular association of the tuberculin type of reaction with infection with living bacteria the reader is referred to the discussions in the preceding paper mentioned above. We may reiterate here, however, that the possibility of development of a specific hypersusceptibility on the part of an infected animal to metabolic products of the bacteria produced in quantity and perhaps slightly diffusible, a susceptibility definitely separable from protein anaphylaxis and developing much sooner in the course of infection, would go far to explain many of the injuries and degenerations occurring in bacterial diseases. Moreover, these observations seem to promise further light upon phenomena of hypersusceptibility in general, in that we appear to have been dealing with a form of specific susceptibility in which not only the laws of sensitization but also the physical and chemical properties of the injuring antigen seem to differ materially from those governing protein anaphylaxis.

It seemed more than likely that we were dealing with a general principle. In the first place, this was indicated by the close analogy, in regard to nearly all the circumstances mentioned above, between the tuberculin reaction and such phenomena as the mallein, typhoidin, and abortin reactions in guinea pigs. In these phenomena it seems fairly well established, in view of the recent work of Fleischner, Meyer, and Shaw (5), that infection and not immunization is necessary for the development of skin hypersusceptibility. Again, the substance studied by us is strikingly similar to the substances studied by Dochez and Avery (6) in 1917 with regard to the pneumococcus and other organisms.

Dochez and Avery obtained a substance specifically precipitable in pneumococcus serum in young broth culture filtrates of pneumococci, in the blood and urine of rabbits experimentally infected with pneumococci, and in the blood and urine of pneumonia patients. Fornet (7), moreover, as early as 1906 observed substances precipitable with antityphoid sera, in the blood and urine of typhoid patients, possibly similar to the material of Dochez and Avery. Ascoli (8), too, found specifically precipitable materials in extracts of old and extensively putrefied spleen tissue of anthrax animals, the condition of the material indicating

that he may have worked with similar antigens. Moreover, work of considerable importance along these lines was done by Pick (9) as early as 1902. Pick found that when he filtered salt solution extracts of 3 day old agar cultures of typhoid bacilli, he obtained specifically precipitable materials which gave no biuret reaction and often no Millon reaction, and did not precipitate with tannic acid, picric acid, or uranium acetate. His material was heat-resistant and was not digested by pepsin or trypsin under suitable conditions.

It became necessary, therefore, to extend our observations, first to the problem of whether materials could be obtained analogous to those studied in the tubercle bacillus extracts from similar preparations of other bacteria of biologically varying species; to obtain more light upon the immunological, chemical, and physical properties of these substances; and to determine, if possible, whether animals infected with the respective organisms could be rendered specifically hypersusceptible to these bacterial derivatives.

The present communication represents the beginnings made in these directions.

I.

Specific Serum Reactions with "Residue" Material.

In order to include organisms as widely at variance with each other as possible, from a biological point of view, the experiments were carried out with tubercle bacilli, pneumococci, *Staphylococcus pyogenes aureus*, influenza bacilli, and typhoid bacilli.

Technique of Antigen Preparation.—The remarks in this section will be restricted entirely to the substances spoken of as the "proteose residue" in our last paper, but in designating which we think it wiser now to drop the word "proteose" and to speak of them only as "residue." The technique described below was uniformly followed in producing these substances, and this may be assumed to have been the manner of production throughout, unless special mention is made of modifications. Two distinct procedures were followed, one in which the bacteria were first grown in suitable broth for varying periods, the broth then filtered free of bacteria and subsequently treated exactly like the extracts described below. These broth preparations were used only in order to determine that the living bacteria rapidly secreted this substance into the broth, as had been found to be the case with tubercle bacilli. But since many of the alcohol-precipitable substances in such broth interfered with clean reactions, most of the experiments were done with extracts prepared as follows:

In order to obtain large masses of bacteria for extraction ordinary tin pie plates of two dimensions were employed, approximately 10½ and 11½ inches in diameter, the large one placed over the smaller one in the form of a Petri dish. The lower pie plate was lined with ordinary wrapping paper, simply by pressing the paper into the plate with another plate, putting them together like Petri dishes, and in this way autoclaving.[1] Hormone agar, blood agar, and chocolate agar were poured into these plates as into Petri dishes, incubated over night, and then used. The plates were inoculated with suspensions from agar slants, spread with glass spreaders, and the bacteria were grown, then collected by scraping off with glass spreaders in salt solution, and centrifugalized down. They were then washed, dried over sulfuric acid, and ground with marbles for from 2 to 3 weeks. With tubercle bacilli, staphylococci, and typhoid bacilli, the grinding seemed absolutely necessary for the obtaining of suitable yields of extracted material. With influenza bacilli and pneumococci, grinding was unnecessary. The ground bacteria were placed into shaking bottles in varying quantities of salt solution, alkalinized to a pH of about 9 to 9.4, and shaken for about 4 hours. They were then either immediately used for further preparation, or were neutralized to pH 7 to 7.4, and placed in the ice chest; the neutralization, as will be seen, was necessary in order to avoid deterioration due to possible racemization of the antigenic substances.

The further treatment, entirely analogous to the preparation of the tubercle bacillus extracts, was as follows: The material, after shaking, was centrifugalized in order to remove gross particles and then filtered through Berkefeld filters. This opalescent filtrate was precipitated by adding 10 per cent acetic acid, drop by drop, in the cold, until the further addition of acid no longer brought down a precipitate. In all cases a flocculent precipitate appeared which was removed by centrifugalization. This fraction is spoken of as either nucleoprotein or phosphoprotein, or both, and will be made the subject of chemical studies to follow. To the opalescent, still acid, supernatant fluid, a little more acid was added to make sure that nothing further came down, and normal NaOH was then added, drop by drop, and the mixture shaken, after each addition, in order to make sure that we had not, by adding excessive acid, redispersed some of the precipitable nucleoproteins by passing beyond the optimum precipitation point. The procedure was continued up to the neutral point, and as it was approached a precipitate usually came down which was rich in phosphates. When the precipitate appeared it was filtered off and any turbidity appearing during the process was in each case removed by filtration. The preparation was then acidified and boiled at the acid reaction for from 3 to 5 minutes. In many instances hardly any coagulable proteins came down. In some, especially with typhoid bacilli, appreciable turbidity developed; this was then removed by filtration through a Berkefeld filter. The filtration was carried out while the material was hot and the filtrate

[1] Lately large glass Pyrex pie plates with tin covers have been found to be more convenient.

was allowed to cool after filtration. As stated in the previous paper, occasionally a very slight haze perhaps due to Bence-Jones protein appeared. Whenever this appeared, it was again removed by filtration.

The resultant material was water-clear and was now neutralized before use, to approximately pH 7; a moderate precipitate again developed which was shown to consist largely of phosphates. This again was removed.

This final material is the so called residue with which we are dealing.

Chemical tests were done with sufficient frequency to show that experiments such as those outlined below could be obtained with every organism used with materials which reacted negatively to the biuret, Hopkins-Cole, Millon, and sulfosalicylic acid protein tests.

All these antigens gave precipitation turbidity with ten volumes of absolute alcohol, which varied in intensity in different preparations. It is not impossible that the alcohol-precipitable material is merely incidental, carrying down the antigen material with it. We say this because antigenic potency did not quantitatively parallel alcohol precipitation.

One of the great difficulties in characterizing the substances studied is the possibility that we were dealing with minute quantities of true protein, too slight to be detected chemically, but still sufficient to give biological reactions. This can be conclusively determined only by actual chemical analysis, a task which has so far been rendered difficult by the very small actual quantities available. It will be interesting to note in this connection that, in several cases, we compared the biuret reaction and alcohol precipitation of antigens which gave powerful specific reactions, with dilutions of horse serum in which the protein concentration was about 1:1,000. In such cases the horse serum solution, which still gave a strong biuret reaction, gave a much fainter alcohol precipitation than the residue antigen, which no longer gave a biuret, a fact which indicates, at least indirectly, that most of the material that comes down with the alcohol is not biuret-reacting protein. Protocols are given below of experiments by which the antigenic properties and the specificity of residue antigens prepared as above, from various bacteria, were investigated.

Experiment 1. Specific Precipitation of Residue Antigens with Specific Antisera.—

Serum.	Residue.	Precipitation.	
Influenza Serum 103	Influenza.	+++ in 3 min.	++++ in 15 min.
	Pneumococcus.	0	
	Staphylococcus.	0	
	Typhoid.	0	
	Tubercle.	0	
Pneumococcus serum (horse).	Influenza.		0
	Pneumococcus.	+++ in 3 min.	++++ in 15 min.
	Staphylococcus.		0
	Typhoid.		0
	Tubercle.		0
Staphylococcus Serum 55	Influenza.		0
	Pneumococcus.		0
	Staphylococcus.	++ in 5 min.	++++
	Typhoid.		0
	Tubercle.		±
Normal rabbit "	Influenza.	0	
	Pneumococcus.	0	
	Staphylococcus.	0	
	Typhoid.	0	
	Tubercle.	0	
" horse "	Influenza.	0	
	Pneumococcus.	0	
	Staphylococcus.	0	
	Typhoid.	0	
	Tubercle.	0	

Typhoid serum and tubercle serum were not included in this series because of the weakness of the sera available at the time. It is unnecessary to duplicate experiments of this kind since they were done again and again with results entirely alike in significance, though with some sera a very faint non-specific reaction was occasionally obtained, always so faint that it did not interfere with correct interpretations.

Experiments with broth filtrates similarly treated are not given since they were essentially the same, except that specificity was not so sharp. The broth filtrate method for general work was abandoned after the appearance of these substances in the broth had been definitely shown.

Experiment 2. Experiment to Determine Whether the Alcoholic Precipitate Really Carries the Specific Precipitable Material.—This experiment was performed because of the occasional heavy serum reaction with material giving a relatively faint alcoholic precipitate.

2 cc. each of pneumococcus, influenza, and staphylococcus residue antigens were precipitated with ten volumes of absolute alcohol, centrifugalized, dried, and taken up in one-half isotonic salt solution—isotonicity controlled on washed sheep cells.

Antigen.	Serum.		Precipitation.
Pneumococcus antigen precipitated with alcohol and redissolved.	+	pneumococcus serum.	++++ immediate.
		influenza "	±
		staphylococcus "	−
Staphylococcus antigen precipitated with alcohol and redissolved.	+	pneumococcus "	±
		influenza "	±
		staphylococcus "	++++
Influenza antigen precipitated with alcohol and redissolved.	+	pneumococcus "	−
		influenza "	++++
		staphylococcus "	−

It is noticeable in this experiment that after alcohol precipitation and resolution the reactions are, if anything, more sharp and specific than before.

Having determined the precipitability of these residue antigens, we next investigated the question of their complement-fixing powers. In regard to the tubercle bacillus, complement fixation by these residues is being studied in our laboratory and at Saranac by Petroff who will report this phase of the work in a subsequent publication.

Experiment 3. Experiment to Determine the Complement-Fixing Properties of the Residue Antigen.—The quantities given in this experiment are based upon a considerable number of titrations, these being necessary since we had, of course, no significant quantitative measure for the amounts of antigen present in the final residues and since the antibody contents of the various sera must have varied enormously. The obvious technical description of complement fixation is omitted. Complement was always titrated in the usual way and about 1.5 to 2 units employed. Preliminary incubation was always for 1 hour, and readings were made by constant observation after the addition of the sensitized cells. ++++, as usual, signifies complete fixation.

Serum.	Antigen.	Amount of antigen.	Complement fixation. Residue antigens.	
		cc.	*20 min.*	
Influenza Serum 520, 0.025 cc.	Influenza.	0.01	++++	
	Staphylococcus.	0.05	0	
	"	0.01	0	0.3
	Typhoid.	0.1	0	1 to 10 compl.
	"	0.2	0	± 2 units.
	Tubercle.	0.1	0	
				1 hr.
Staphylococcus serum (rabbit) 0.025 cc.	Influenza.	0.01	0	0
	Staphylococcus.	0.05	++++	++++
	"	0.01	+++	+++
	Typhoid.	0.1	+	+
	"	0.2	+++	+++
	Tubercle.	0.1	++++	++++
Typhoid Serum 1043 (rabbit) 0.05 cc.	Influenza.	0.01	0	0
	Staphylococcus.	0.05	0	0
	"	0.01	0	0
	Typhoid.	0.1	++++	+++
	"	0.2	++++	++++
	Tubercle.	0.1	++++	++++
Tubercle bacillus serum (sheep) 0.05 cc.	Influenza.	0.01	0	0
	Staphylococcus.	0.05	0	0
	"	0.01	0	0
	Typhoid.	0.1	±	0
	"	0.2	±	0
	Tubercle.	0.1	++++	++++
Normal rabbit serum 0.025 cc.	Influenza.	0.01	0	
	Staphylococcus.	0.05	0	
	"	0.01	0	
	Typhoid.	0.1	0	
	"	0.2	+	
	Tubercle.	0.1	+	Complete hemolysis in 12 min.
Normal sheep " 0.05 cc.	Influenza.	0.01	0	
	Staphylococcus.	0.05	0	
	"	0.01	0	
	Typhoid.	0.1	0	
	"	0.2	0	
	Tubercle.	0.1	0	

In this experiment some non-specificity is apparent. The staphylococcus serum, while it fixed with staphylococcus in quantities as low as 0.01 cc., fixed with larger quantities of typhoid and tubercle antigen. It also gave a ++++ fixation with tubercle antigen. However, there was a + fixation only of this serum with as much as 0.1 cc. of typhoid antigen. The typhoid serum also fixed with the tubercle antigen. It is noteworthy that the tubercle bacillus serum did not fix with either the typhoid or the staphylococcus antigen.

It is not at all unlikely, as subsequent modifications of technique have shown, that these non-specific reactions may be eliminated by more careful quantitative determinations. With the influenza antigen we never had the slightest difficulty, since it was always absolutely specific, in some experiments fixing in quantities as low as 0.001 cc. of the residue which gave only a slight turbidity on alcohol precipitation. This experiment with all its defects is cited since it illustrates the kind of irregularity to be expected, and yet definitely indicates that the principle of the presence of specific antigenic substances in the residues may be maintained.

A number of similar experiments were carried out with redissolved alcoholic precipitates of the residue materials as antigen. In order to save space the experiments are not cited in protocol, but we may state that in an experiment carried out on May 24, 1922, in which the same sera were used and the redissolved alcoholic precipitates of the same antigens employed in the above experiment were used, results entirely analogous to those above cited were obtained, except that the non-specific fixation of the tubercle antigen with the staphylococcus rabbit serum was to a large extent eliminated.

It is important to mention in passing that we have noticed in a number of cases that, in spite of uniform lack of either antihemolytic or hemolytic properties on the part of the antigen, the smaller amounts of antigen, that is amounts of 0.05 and 0.01 cc., gave better fixation than the larger amounts. This cannot be explained at present and must be subjected to further investigation.[2]

[2] It is also of the utmost importance that we should mention our uniform failure to obtain fixation with the pneumococcus residue and pneumococcus horse or bovine serum, in spite of many attempts and in spite of the fact that the pneumococcus antigen was heavily precipitated by both these sera. This might seem to contradict the opinions expressed by one of the writers in a recent publication as to the identity of the complement-fixing and precipitating antibodies and, of course, will be investigated. But it has been found in all these experiments that both the horse and bovine antipneumococcus sera used, possessed to a powerful degree the auxilytic effect described in former years by Manwaring (Manwaring,

II.

Experiments on the Stability of the Residue Antigen.

Early in the experiments the fact that boiling in acid did not destroy the skin-reactive capacity of the residue antigen from tubercle extracts led to the further investigation of the stability of these antigenic substances as obtained from other bacteria. Residue materials were collected from the various organisms which were being studied, and were subjected to serological test to determine their specific reaction capacities. A number of experiments were then carried out to ascertain whether or not they were destroyed by the application of heat at various hydrogen ion concentrations. The following experiment illustrates the first method carried out for this purpose.

Experiment 4. Stability of the Antigenic Material on Boiling in Alkaline and Acid Reactions.—All the antigens described above were precipitated against specific and heterologous sera and found to give specific precipitates. In every case, two 5 cc. fractions of each of the antigens were placed into small 50 cc. Erlenmeyer flasks and brought, respectively, to a pH of 5.4 and 9.4. Each specimen was then boiled in a reflux condenser for 1 hour and at the end of this time they were titrated to ascertain that the pH had not changed and precipitates, which developed in almost every case and will be noted specifically below, were then removed by rapid centrifugalization until the supernatant fluid was clear. These were then brought to pH 7 to 7.2 by careful titration, and precipitation tests were set up. The above description applies to each of the experiments below.

W. H., *Centr. Bakt., Ite Abt., Orig.*, 1906, xlii, 75), an effect like the conglutinin phenomenon of Bordet and Gay (Bordet, J., and Gay, F. P., *Ann. Inst. Pasteur*, 1906, xx, 467), and this is most likely to account for the lack of parallelism between complement fixation and precipitation in these cases. The larger the amount of serum used, the more prompt was the hemolysis, although the serum itself had no sensitizing effect upon red blood cells. This point seems to be of sufficient theoretical importance to be investigated as soon as we can produce a sufficiently strong antipneumococcus rabbit serum and analyze the apparent conglutinin effect of several specimens of antipneumococcus horse serum which we have noticed and which is particularly curious in face of the fact that, so far as we know, conglutinins have not been described in horse serum.

Comparative Precipitation Tests on Staphylococcus Antigens, Original and Boiled for 1 Hour at pH 9.4 and 5.4.

Material.	Precipitation.
Staphylococcus antigen (original) + staphylococcus serum.....	++++
" " " + influenza "	−
" " " + pneumococcus "	+ to ++
" " (boiled 1 hr. at pH 9.4) + staphylococcus serum..	−*
" " (" 1 " " pH 9.4) + influenza "	−*
" " (" 1 " " pH 9.4) + pneumococcus "	+ to ++*
" " (" 1 " " pH 5.4) + staphylococcus "	+++
" " (" 1 " " pH 5.4) + influenza "	−
" " (" 1 " " pH 5.4) + pneumococcus "	+

* This was repeated with like result.

This experiment shows that the antigenic properties of the staphylococcus substance are destroyed by prolonged boiling (perhaps racemized) at pH 9.4, but remain intact for 1 hour at an acid reaction of 5.4. The slight non-specific reactions are not effected by this treatment.

Comparative Precipitation Tests on Influenza Antigens, Original and Boiled for 1 Hour at pH 9.4 and 5.4.

Material.	Precipitation.
Influenza antigen (original) + influenza serum.....	++++
" " " + pneumococcus serum.....	+ to ++
" " " + staphylococcus "	−
" " (boiled 1 hr. at pH 9.4) + influenza serum.....	−
" " (" 1 " " pH 9.4) + pneumococcus serum.....	+
" " (" 1 " " pH 5.4) + influenza "	++++
" " (" 1 " " pH 5.4) + pneumococcus "	± to +

This series shows that, as with the staphylococcus antigen, boiling in an alkaline reaction destroys antigenic properties of the influenza substance, while similar treatment with acid has no effect.

Comparative Precipitation Tests on Pneumococcus Antigens, Original and Boiled for 1 Hour at pH 9.4 and 5.4.

Material.	Precipitation.
Pneumococcus serum + original pneumococcus antigen.....	++++
" " + pneumococcus antigen at pH 9.4 over night.....	++++
" " + " " " pH 9.4 in reflux condenser boiled 1 hr.....	++++
" " + pneumococcus antigen at pH 5.4 in reflux condenser boiled 1 hr.....	++++

This series shows that the pneumococcus substance, like the tubercle bacillus material, is more resistant than either the staphylococcus or the influenza residue.

Having thus shown that boiling in a reflux condenser[3] for 1 hour at an acid reaction does not materially change any of the antigens employed, but that boiling at pH 9.4 destroyed the specific precipitability of the influenza and staphylococcus antigens, we carried out similar experiments in which the severity of the procedure was increased by subjecting the residue antigens to autoclave digestion. The two antigens were used which were apparently the most susceptible to boiling with alkali to see whether in an acid reaction which did not affect them after boiling in a reflux condenser, they could be destroyed under conditions of increased pressure.

[3] During the process of boiling in a reflux condenser both in the acid and alkaline specimens, but chiefly in the acid, rather heavy precipitates came down. These were removed by centrifugalization and proved by the molybdate test to contain copious phosphates. Removal of these precipitates did not seem to detract in any way from the specific precipitability of the material, and it suggests the possibility of, in this way, further purifying the antigen. In the experiment with the staphylococcus antigen boiled 1 hour at pH 9.4 the suspicion arose that possibly the antigenic properties which had been removed from the solution might have come down with the precipitate. For this reason, the precipitate was redissolved in salt solution and tested against serum, but with negative results, showing that this was not merely a precipitation of antigenic properties. It was also to be noted that, although alkaline hydrolysis removed antigenic properties from the influenza and staphylococcus antigens, it did not change the slight non-specific reactions which these antigens gave with the pneumococcus horse serum.

Experiment 5. Effect of Autoclave Digestion on the Precipitin and Complement-Fixing Reactions of the Influenza and Staphylococcus Antigens.—Antigens used on the previous day, Influenza Residue A and staphylococcus residue, were set up for precipitin and complement-fixing reactions in the morning. The residues of these antigens, plus pneumococcus antigen, were brought to the following reactions, the divergence in pH being due to the necessity of working with small quantities and letting well enough alone: influenza 5.4, pneumococcus 5.8, and staphylococcus 5.2. These materials were then autoclaved at three atmospheres for 1 hour, the actual three atmospheres being at 145°C. for 40 minutes. They were removed from the autoclave, neutralized from pH 7.2 to 7.4, and precipitation tests were again set up, also complement fixation tests, as follows:

Precipitation Tests.

Material.	Precipitation.	
	Before autoclaving.	After autoclaving.
Normal Serum 1 + Influenza Residue A	0	0
" " 1 + staphylococcus residue	+	0
" " 2 + Influenza Residue A	0	0
" " 2 + staphylococcus residue	±	0
Influenza " 520 + Influenza Residue A	++++	0
" " 520 + staphylococcus residue	+	0
Staphylococcus Serum 55 + Influenza Residue A	0	0
" " 55 + staphylococcus residue	+++	++

Complement Fixation Tests.

Residue antigens.	Influenza Serum 520, 0.025 cc.	Staphylococcus Serum 55, 0.025 cc.	Normal Rabbit Serum 1, 0.025 cc.	Normal Rabbit Serum 2, 0.025 cc.
Before autoclaving.				
0.01 cc. of Influenza Antigen A	++++	0	0	0
0.001 " " " " "	++++	0	0	0
0.1 " " staphylococcus antigen	0	++++	±	0
0.01 " " " "	0	+	0	0
After autoclaving.				
0.01 cc. of Influenza Antigen A	0	0	0	0
0.001 " " " " "	0	0	0	0
0.1 " " staphylococcus antigen	0	++++	0	0
0.01 " " " "	0	++	0	0

This experiment, then, illustrates the fact that apparently the more delicate antigen, the influenza one, is altered under a pressure of three atmospheres in an acid reaction, whereas the staphylococcus antigen, being more resistant, is slightly diminished in antigenic potency but not destroyed.

A similar experiment, in which the pneumococcus and the staphylococcus residues were used, is the following.

Experiment 6. Effect of Autoclave Digestion on the Precipitin Reaction of the Staphylococcus and Pneumococcus Antigens.—Staphylococcus and pneumococcus residues prepared 5 days previously were precipitated against the respective sera as follows:

Staphylococcus serum +	staphylococcus residue ++++ within a few minutes; strong ring formation.
	pneumococcus residue negative after 2 hours.
Pneumococcus serum +	staphylococcus residue negative after 2 hours.
	pneumococcus residue ++++ almost immediately; powerful ring formation.

These antigens, about 2.5 cc. of each, were acidified with a drop of 10 per cent acetic, and now turned litmus strongly red. Not titrated because available volume too small. Autoclaved at four atmospheres for 40 minutes.

Neutralized by careful addition of weak soda till both about pH 7.4.

Again set up as above with like result except that the precipitin with the homologous staphylococcus tube comes out much more slowly and weakly than before autoclaving; takes about 10 minutes to be distinct, but then gradually deepens. That in the pneumococcus tube comes out as rapidly and distinctly as before. Specificity apparently not impaired by the autoclave treatment.

This experiment shows that in an acid reaction the pneumococcus residue is not altered to the slightest degree by subjection to a pressure of four atmospheres for 40 minutes in an autoclave.

Similar experiments were done with these antigens on repeated occasions and in general it was shown that the precipitability and specificity of the pneumococcus and tubercle bacillus residues were not affected by acid and autoclaving; the staphylococcus residue, however, was definitely altered and the influenza residue was completely deprived of its antigenic properties by similar treatment.

These experiments, as well as observations upon the preservation of the antigens in the ice box, indicated that, whereas the residues were all extremely resistant to treatment by heat in acid reactions, they were to some degree susceptible to similar treatment when the reaction was alkaline. The suggestion that racemization might account for this is, of course, an obvious one, and for this reason

an experiment was done in which the antigens were rendered alkaline to a pH of 9.4, and preserved in this condition in the ice chest and tested from day to day.

Experiment 7. Rapidity of Deterioration of the Residue Antigens at pH 9.4 at Low Temperatures.—Antigens of influenza, pneumococcus, staphylococcus, and tubercle bacilli prepared as usual by alkaline extraction, subsequent precipitation of acid-precipitable material, filtration, boiling in acid state, and refiltration. All of these brought to pH 7 to 7.2, and precipitated as follows:

Material.	Precipitation.
Influenza serum + influenza antigen	++++
" " + pneumococcus antigen	−
" " + staphylococcus "	−
" " + tubercle "	−
Pneumococcus serum + influenza "	+ to ++ slow.
" " + pneumococcus antigen	++++ immediate.
" " + staphylococcus "	+ to ++ slow.
" " + tubercle "	Not done.
Staphylococcus " + influenza "	−
" " + pneumococcus "	−
" " + staphylococcus "	++ to +++ slow.
" " + tubercle "	Not done.

Tubercle bacillus serum not done with other antigens because too little serum available. This controlled in another experiment done on same day.

Tubes of each antigen brought to pH 9.4, and set away in ice chest. Specimens removed at 24 hour intervals on May 17, 18, and 19, 1922, 12, 24, 48, and 72 hours, respectively; such specimens in each case brought to pH 7 and 7.2 and precipitations done.

May 17. After 24 hours all precipitated.

May 18. After 48 hours influenza antigen no longer reacted, but all others still active as before.

May 19. After 72 hours, staphylococcus, pneumococcus, and tubercle antigens still precipitable and specificity tests done and found as above.

May 22. After 6 days tubercle and pneumococcus antigens again tested and are still precipitable by their respective sera, not noticeably less than at first. (No staphylococcus antigen left.)

This experiment, then, shows that the influenza antigen deteriorates within 48 hours at a reaction of pH 9.4, whereas neither pneumococcus, tubercle bacillus, nor staphylococcus is affected within 74 hours by such treatment, and the tubercle antigen, as well as the pneumococcus,

as was to be expected, retained activity for as long as 6 days. It is apparent that except for the influenza antigen, alkali has no very powerful deteriorating effect upon the residues. Whether or not the powerful effect of alkali upon the influenza, and its definite but less powerful effect upon the staphylococcus residue are due to a possible racemization, we cannot say until we can work with larger quantities of these materials.

Does the Residue Antigen Incite Antibody Formation in Animals?

It is thus plain that the residue of bacterial extracts from which all coagulable proteins have been removed as completely as this can be done, is still capable of reacting specifically with antisera and that this is true of a considerable number of different bacteria of widely varying biological properties. As far as the pneumococcus is concerned, we believe that the substance described by Dochez and Avery is the same which we have isolated by extraction of the bacterial bodies and that it is analogous to the tuberculin substance that we obtained by this method last year and with which we carried out the work on skin reactions then reported.

These residue substances, then, are antigens at least in the sense that they will react with specific antibodies. Are they antigens also in the sense that they may induce the formation of antibodies? This, of course, was a very obvious line of inquiry and we can briefly summarize our knowledge, in this regard, by stating that up to the present time no one in our laboratory has succeeded in producing specific antibodies by the injection of the residue antigens. From this we know definitely that the residue antigens do not induce antibody formation when injected into animals with the same ease with which this can be accomplished with whole bacteria or bacterial extracts. Until more extensive animal immunization with these materials has been attempted, we would not venture to claim that antibodies are not induced by them at all. It may be, of course, that up to the present time we have not been able to inject sufficiently large quantities. On the other hand, our failure may be due to the rapid elimination of these substances in the urine, as with the pneumococcus substances described by Dochez and Avery. Attempts to induce antibody for-

mation are being continued. Again, it is at least worth speculating upon the possibility that these substances may represent the so called "*Haptenes*" foretold by Landsteiner (10) and characterized as capable of reacting with antibodies without being able to induce antibody formation. In a recent paper on the chemical modification of proteins and the antigenic alterations so obtained, Landsteiner speaks of such substances, and suggests in his final reasoning that tuberculin may fall into this category. It is at least possible that in the materials described above we are dealing with substances of this kind.

It is not impossible that the work of Meyer (11), who observed that certain tapeworm lipoids were incapable of inducing complement-fixing antibodies on injection into animals, although they furnished a good antigen for specific complement fixation, is pertinent in this connection. This, however, is not certain because in reactions in which lipoidal materials were used we may be dealing with phenomena comparable to the Wassermann reaction. However this may be, there seems to be very little doubt about the complete correspondence of our results with those of Pick (9). Pick mentions the fact that the biuret-free bodies obtained by trypsin digestion of a typhoid extract, which were still precipitable by specific serum, seemed to have lost their property of antibody formation upon injection into the animal, and he (9) states in unqualified terms that he believes that the properties of inducing antibody formation in the body and the phenomena of specific precipitability *in vitro* may not be entirely parallel attributes.

III.

Experiments on Animals.

Since, from experiments like those cited above, it was plain that the residue antigens of bacteria could be specifically precipitated and were specifically capable of fixing complement with antibodies, it was necessary to see whether such specificity extended to their reactions with the infected animal body. The experiments in this direction are not complete and are being confirmed, but it has seemed wise to add to this paper information along these lines as far as we have obtained it up to the present time.

The first question we attempted to answer was whether specific residue antigens prepared from bacteria other than the tubercle bacillus would give reactions with tuberculous animals which were sensitive to the tubercle bacillus residue. The following experiments are examples of a considerable number done in order to elucidate this point.

Experiment 8. Specifity of Skin Reactions Tested with Tubercle Bacillus Residue.—The antigens used in this experiment had all been previously tested by precipitation and complement fixation and had been found to be specific. They all consisted of residues prepared by the general method described at the beginning. They were intracutaneously injected in quantities of approximately 0.1 cc. into each of four guinea pigs, two tuberculous animals and two normals. The results were as follows:

Animal No.	Antigens.			
	Tubercle.	Staphylococcus.	Influenza.	Pneumococcus.
Tuberculous guinea pigs.				
1	+++	−	−	±
2	+++	−	−	−
Normal controls.				
3	−	++	−	++
4	±	+	±	−

A similar experiment performed under conditions identical with the above gave the following results.

Experiment 9. Specificity of Skin Reactions Tested with Tubercle Bacillus Residue.—In this experiment the residue antigens used for complement fixation on the preceding day were used as prepared for tubercle bacilli, influenza bacilli, pneumococci, and staphylococci, respectively. They were intracutaneously injected in quantities of approximately 0.1 cc. into each of five guinea pigs, two injections being given under either flank. The results were as follows:

Animal No.	Antigens.			
	Staphylococcus.	Tubercle.	Influenza.	Pneumococcus.
Tuberculous guinea pigs.				
5	−	++	−	++
6	−	++	−	+
Normal controls.				
7	−	−	−	++
8	−	−	−	+++
9	−	−	−	++++

It is apparent from these experiments and others like them that the general specificity of the antigens holds good in the animal body as well as *in vitro*. The tuberculous animals, while distinctly susceptible by intradermal test to the tubercle bacillus residue, do not react specifically to antigenic residues from other bacteria. It is interesting to note also that the pneumococcus residue antigen possessed, in these experiments as in some others, a not inconsiderable degree of non-specific toxicity for normal animals, and that to a very slight degree this was also true of the staphylococcus antigen in a few animals. This point will be referred to later.

The next point of importance was to find out by actual experiment whether the converse of the above conditions was true; namely, whether specific skin hypersusceptibility to residue antigens could be induced in guinea pigs infected with the other bacteria mentioned above.

To a considerable extent an affirmative answer to this question has been furnished by the work of previous investigators, particularly of recent years by the important contributions of Fleischner and Meyer (12), and of Fleischner, Meyer, and Shaw (5). It has, of course, been known for a long time that there is a striking analogy between the tuberculin and the mallein reactions, mallein being produced in a manner analogous to that of old tuberculin and very probably containing physically and chemically comparable active substances. They found that the laws governing such reactions in *abortus*-infected animals were completely analogous to those revealed for tuberculous guinea pigs, especially by Baldwin and Krause; namely, that the reaction is associated with actual infection and that even very intensive immunization with dead bacilli or the proteins derived from them never induced typical skin reactions. In some of their tests the material used for intradermal injection consisted of 8 week glucose broth

cultures of the organisms heated to 100°C. for 1 hour, a procedure which makes it likely that their materials were analogous to our own. In a later communication, Fleischner, Meyer, and Shaw (5) add a considerable number of their own experiments to a valuable review of skin hypersusceptibility in which they again note this difference in regard to skin hypersusceptibility between infected and immunized animals, not only for *B. abortus bovinus*, but for the typhoid bacillus, in the case of which they sometimes obtained temporary cutaneous hypersensitiveness after the intravenous injection of living bacilli.

This universal agreement in regard to the differentiation between cutaneous hypersensitiveness and true anaphylaxis, with our own analysis of the conditions in tuberculosis, would seem to indicate that a generalization of our ideas concerning the specific susceptibility of infected animals to the residue materials might almost be justified. In spite of this close analogy, however, we determined to continue the experiments in the hope of obtaining specific skin hypersensitiveness to the residue antigens in animals infected with the various organisms employed in the general investigation.

The great difficulty in such experiments is the fact that, while it is quite easy to obtain subacute and chronic infections in guinea pigs with tubercle bacilli, it is a difficult task to produce similar reasonably prolonged infections in these animals with the other bacteria. The animals in most cases either recover rapidly, or die acutely within a few days, and if repeatedly injected with small doses they are apt to become immune. Moreover, when a noticeable degree of skin hypersusceptibility in such infections is attained, it is apt to be more short lived than the state of anaphylactic protein hypersusceptibility, a point that Meyer mentions, that appears from Krause's work concerning the parallelism between the activity of a focus and skin sensitiveness, and which also was apparent in some of our own work. We may mention in passing that in our first paper, recognizing the apparent dependence of skin hypersusceptibility to actual infection, we attempted to imitate the conditions of absorption from an active focus of bacteria by treating guinea pigs with large injections of nucleoproteins, hoping that the gradual breaking down of these materials might sensitize in a manner similar to that obtained in the infected animal. More recently, with Petroff, we have carried on such studies, both with the nucleoprotein fraction and with killed tubercle bacilli,

with degrees of success which will be published later. In these experiments, too, the transitory nature of the typical skin reactiveness has been noticed. The reactions, therefore, must be done at a very definite time in order to yield positive results.[4]

We tried a great many different methods of producing infections in guinea pigs. None of these methods, however, have been uniformly successful and it would, therefore, needlessly prolong our communication to go into details about all of them. The following experiment is perhaps the best so far obtained in which specific intracutaneous reactions observed to staphylococcus residue material were obtained in guinea pigs infected with *Staphylococcus pyogenes aureus*. In these staphylococcus experiments the method employed was a modification of that described by one of the writers with Raymond some time ago (13). Small celloidin capsules were filled with staphylococcus-infected agar; small holes were left in the capsules which were then dropped into the peritoneal cavity of the animal.

The following is one of the experiments done with animals prepared in this way.

Experiment 10. Specificity of Skin Reactions Tested with Staphylococcus Residue. —June 29, 1922. Six guinea pigs were operated upon under ether anesthesia and capsules filled with agar, into which staphylococcus had been inoculated and in which a considerable number of holes had been left, were tucked into the peritoneal cavity. Three of these guinea pigs died between June 29 and July 10. July 10. Skin reactions were done on survivors, as well as on tuberculous and normal controls. These tests were repeated on July 13, readings in each case being made after 24 hours.

The staphylococcus material used in this experiment was made from staphylococcus powder that was quite old and apparently had begun to putrefy. It had

[4] We would like to call attention here to a curious fact which has caused us a great deal of difficulty and may be of interest to others working with skin reactions in guinea pigs. We have found that when very young guinea pigs were used, animals under 250 gm., intracutaneous reactions of all kinds were apt to give irregular results. Whether this is due to the difficulty of applying the dose entirely into the superficial layers of the skin in thin skinned animals, or whether there is a physiological reason for these inconsistencies, we cannot say. Bacteriologists engaged in the titration of diphtheria antitoxin by the Römer method have told us that they have noticed similar irregularities in young guinea pigs and are using, habitually, animals of 350 to 400 gm. or over for intracutaneous work.

an unpleasant odor and in solution was alkaline. However, prepared in the usual manner, it precipitated powerfully with staphylococcus sera.

Animal No.	Tests of July 10, 1922.		Tests of July 13, 1922.	
	Staphylococcus antigen.	Tubercle antigen.	Staphylococcus antigen.	Tubercle antigen.
Staphylococcus guinea pigs.				
10	+ strong.	−	++	−
11	+++	−	+ strong.	−
12	+ to ++	−	July 11. Died.	
Control tuberculous guinea pigs.				
13	+	++	−	+++
14	+	++ to +++	+	+
Normal controls.				
15	−	−	±	−
16	++ (no redness; diffuse area of edema).	−	−	−

In these experiments, then, a distinct but relatively feeble reaction to the staphylococcus residue antigen was obtained in some staphylococcus-infected guinea pigs about 11 days after injection. The animals were very sick at this time and our general impression was that the reactions were done too late. On July 14, the first two staphylococcus guinea pigs listed in this series were each given 2 cc. of residue antigen intraperitoneally, 4 cc. being given at the same time to two normal controls somewhat lighter in weight than the staphylococcus guinea pigs. The staphylococcus animals were quite sick, but did not die.

The next experiment is similar in significance to the above, except that because of the feebleness of the reactions above indicated, we determined to test not only with staphylococcus residue antigen, but with the nucleoprotein fraction as well. In the work with tuberculosis although the residue antigen gave excellent skin reactions, somewhat stronger reactions had always been obtained by using the nucleoprotein fraction and it had never been possible to free this nucleoprotein fraction of its skin-reactive properties, however frequently it was precipitated and redissolved. The relationship between this nucleoprotein fraction and the residue is not clear. We

believe from certain experiments which will be reported separately that one possible explanation may be the fact that the nucleoprotein fraction represents the mother substance of the residue. This is a question which we cannot enter into in the present connection. Thinking, however, that perhaps the animals were but feebly sensitized and that a more powerful reaction substance might bring out a more definite result, we used both redissolved staphylococcus nucleoprotein and residue antigen in the next experiment, as follows:

Experiment 11. Specificity of Skin Reactions Tested with Staphylococcus Residue and Staphylococcus Nucleoprotein.—

Animal No.	Tests of July 17, 1922.			Tests of July 24, 1922.	
	Staphylococcus residue.	Staphylococcus nucleoprotein.	Tubercle residue.	Staphylococcus residue.	Staphylococcus nucleoprotein.
Staphylococcus guinea pigs; capsule inserted June 29, 1922.					
17	−	+	−	July 22. Died.	
18	−	+++	−	−	−
Capsules inserted July 12, 1922.					
19	+	+++	−	−	−
20	+	+++	+	+++	+++
21	−	+	−	July 20. Died.	
Tuberculous guinea pigs.					
22	+	+	++		
6	−	+	++++		
Normal controls.					
23	±	±	+		
24	−	+	+		
No. 25. Staphylococcus guinea pig; capsule inserted July 20, 1922.				−	−
No. 26. Normal guinea pig.				−	−

Whatever may be the relationship between the nucleoprotein fraction and the residue, a problem for which we have not yet a definite answer, this experiment shows that, just as in tuberculous

guinea pigs, the nucleoprotein fraction gives a more powerful response than does the residue antigen, but in one staphylococcus guinea pig (No. 20) a definite residue antigen response was obtained 12 days after the capsule was inserted. This same guinea pig gave a staphylococcus nucleoprotein reaction a week earlier than this when the residue reaction was very faint. This, too, would indicate that the nucleoprotein reaction was due to its possible contents of the residue material rather than representing a true nucleoprotein reaction, since, in that case, it would mean active sensitization in 5 days. However, this question needs further investigation. Fig. 1 represents a drawing made of Guinea Pig 20 on July 24, showing what we consider to be +++ reactions with the staphylococcus residue and nucleoprotein antigens.

We add one other experiment in which apparently specific reactions were obtained in guinea pigs sensitized by injection of living typhoid bacilli. It is necessary to state that this is the only frankly positive experiment of three made with the typhoid bacillus; but it seems worth citing because the result was definite, and until we find a better method of rendering the skin of guinea pigs reactive to typhoid bacilli, it will not be easy to multiply these results.

Experiment 12. Specificity of Skin Reactions Tested with Typhoid Residue.—Four guinea pigs ranging from 300 to 450 gm. were injected intracutaneously with one-twentieth of a slant of the Rawlings typhoid strain on May 11, 15, 19, 23, and 29, 1922. June 6. These guinea pigs, together with two normal controls, were intracutaneously injected with the residue antigen of tubercle bacilli frequently described in these communications and a similar residue antigen of typhoid bacilli made in the identical manner.

Animal No.	Tests on June 7, 1922, a.m.	
	Typhoid residue antigen.	Tubercle residue antigen.
Typhoid guinea pigs.		
27	+ to ++	–
28	±	–
29	++	–
30	+++ to ++++	–
Normal controls.		
31	+	–
32	±	–

This experiment seemed to show definitely that it is possible by repeatedly inoculating guinea pigs with living typhoid bacilli to produce a condition in which they will react by specific skin reactions to typhoid residue antigen and remain normal to similar injections of the tuberculin antigen. Fig. 2 is a drawing made from the tests on Guinea Pig 30 of this experiment.

As stated above, the work on the production of specific skin hypersusceptibility to residue antigen in guinea pigs is still going on and it will be reported more extensively at a later date. The above experiments have been added to this paper, however, in order to indicate the general trend of the work and to bring the subject up to date, as far as we have been able to investigate it.

SUMMARY.

When filtered alkaline extracts of pulverized bacteria of several varieties are precipitated with acid in the cold, boiled with acid, and all materials thrown down by these procedures removed, there remains a small amount of an alcohol-precipitable material which no longer gives any of the ordinary chemical reactions for proteins, such as the biuret, Hopkins-Cole, Millon, and sulfosalicylic acid reactions. The only protein reaction usually given by this material is a very weak xanthoproteic reaction. Nevertheless, the material, which is, as far as we can determine at present, free from coagulable protein, is specifically precipitable by homologous antiserum and gives specific complement fixation reactions.

Such material can also be obtained from organisms like the influenza bacillus, pneumococcus, and meningococcus by extraction without preliminary grinding of the bacteria, and is present in filtrates of young and old broth cultures of the organisms.

We believe that these acid- and heat-resistant antigenic materials are analogous to tuberculin and to the pneumococcus substances with which Dochez and Avery (6) made their observations some years ago.

The stability of these substances is considerable and was investigated particularly because we thought this represented an indirect method of eliminating the possibility of their protein nature. In all cases boiling in a reflux condenser at an acid reaction ranging from pH 5 to 6 for 1 hour failed to destroy the antigenic specificity of the

residue antigens. After such treatment satisfactory and specific precipitation reactions could be obtained. Similar boiling in alkaline reactions, however, destroyed the precipitability of staphylococcus and influenza residues. Subjected to autoclave digestion at an acid reaction of pH 5.4 for 1 hour at from three to four atmospheres, none of the antigenic residues investigated, except that obtained from the influenza bacillus, were destroyed. The pneumococcus and tubercle bacillus residue antigens were resistant to boiling for 1 hour, both in acid and alkaline reactions (pH 5.4 and 9.4). In fact, none of the procedures resorted to made any difference with these two last mentioned substances. It would seem that these facts would add considerable weight to the assumption that the materials dealt with were not ordinary whole proteins.

On preservation in the ice box at an alkaline reaction of pH 9.4, the influenza residue deteriorated within 48 hours, but the other antigens withstood similar treatment for 6 days.

In spite of the fact that these residue antigens were precipitable by homologous sera produced by immunization with the whole bacteria or their unfractionated extracts, we have so far failed to produce antibodies in animals by injecting these residues. While this may be due to inability to inject sufficient amounts of the material it still suggests strongly the possibility that we may be dealing with substances that are antigenic only in the sense that they are able to react with antibodies, but are themselves incapable of inciting antibody production. We suggest, in this connection, the possibility of the relationship between the power of antibody production and molecular size. This phase of the work is being continued on a more extensive scale.

Our work on the reactions of the residue materials in infected animals indicates, as far as we have gone, that complete analogy exists in this respect between the conditions prevailing in guinea pigs infected with these organisms and those previously elucidated for tuberculous animals. This is in keeping with previous knowledge concerning the analogies between the mallein and tuberculin reactions and the studies on skin hypersusceptibility in *Bacillus abortus*- and typhoid-infected guinea pigs reported by Meyer and his coworkers.

It would seem from all these facts that, in guinea pigs infected with bacteria capable of forming foci in the body, infection is followed within a variable, but relatively short time (5 days to 2 weeks) by a type of hypersusceptibility which is distinct from protein anaphylaxis and which may be determined by intradermal skin reaction. It appears likely that the growing bacteria elaborate in the animal body a metabolic product, possibly not a whole protein, which, though practically non-toxic to normal animals, may become highly and specifically injurious to the infected ones.

Such a conception, if further confirmed, would lead to greater clearness in our comprehension of the toxic effects occurring in infections with organisms not true exotoxin producers and, judging by the cellular injuries observed in severe skin reactions, may easily explain focal necrosis and the deeper cellular degenerations observed in the course of many bacterial diseases.

The general bearing of this work upon conceptions of hypersusceptibility is obvious and has been briefly discussed in another paper. Its chief significance is in holding out the hope that we may be able to elucidate the mechanism of a type of specific hypersusceptibility in which the antigen concerned is not a coagulable protein and in which the laws of sensitization in regard to time and quantity differ from those recognized in true protein anaphylaxis.

It seems likely that a recognition of the fact that physical and chemical differences in the substances leading to various forms of specific hypersusceptibilities in the animal body must necessarily influence the mechanism of sensitization, may furnish a clue to further investigations. As such materials become simpler in structure, they fail to induce typical antibody production and by gradually increased diffusibility transfer the reactions from the cell surface to the interior of the cell. The extremes of the scale of differences would be represented by protein anaphylaxis, on the one hand, and drug idiosyncrasies, on the other. Although this suggestion is largely speculative, it has seemed worth mentioning as a line of reasoning suggested by our work.

Incidentally, these studies may indicate the usefulness of the residue antigens for specific precipitation and complement fixation reactions for routine purposes in laboratory investigations.

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EXPLANATION OF PLATE 14.

FIG. 1. Guinea Pig 20. July 24, 1922. Experiment of July 23 to 24. This animal was one of two out of six left alive, into the peritoneal cavities of which perforated celloidin capsules filled with staphylococcus-infected agar had been placed on July 12. For previous records of this animal see the other experiment in the text. The point marked 1 is the reaction appearing after 24 hours at the site of the intracutaneous injection of 0.1 cc. of the residue antigen prepared as usual. At the point marked 2 there was a similar reaction with the so called nucleoprotein fraction.

FIG. 2. Guinea Pig 30. Tested on June 7, 1922. This animal had received five intracutaneous injections of living typhoid bacilli and was tested with residue antigens from the tubercle bacillus and from the typhoid bacillus on the 8th day after the last injection. The spot marked 1 represents the reaction, after 24 hours, to the injection of tubercle bacillus residue antigen. The spot marked 2 represents the point at which the typhoid bacillus residue antigen was injected.

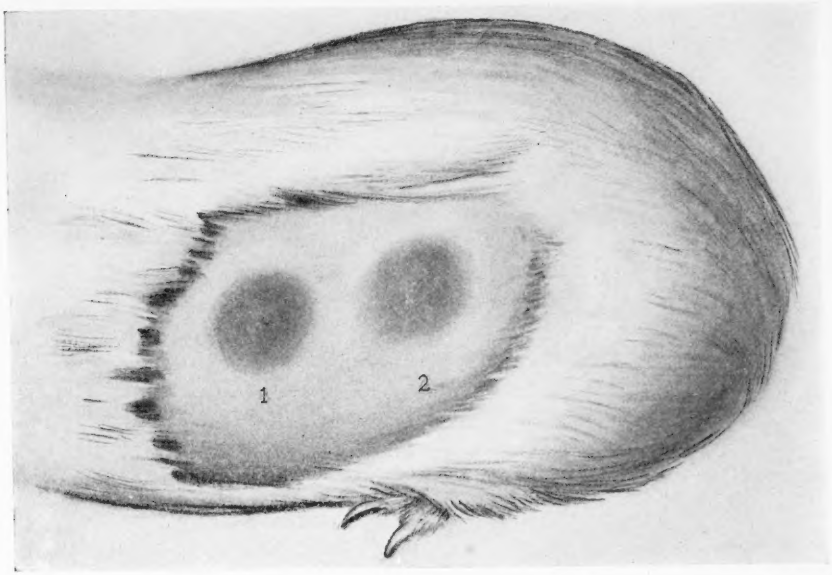

FIG. 1.

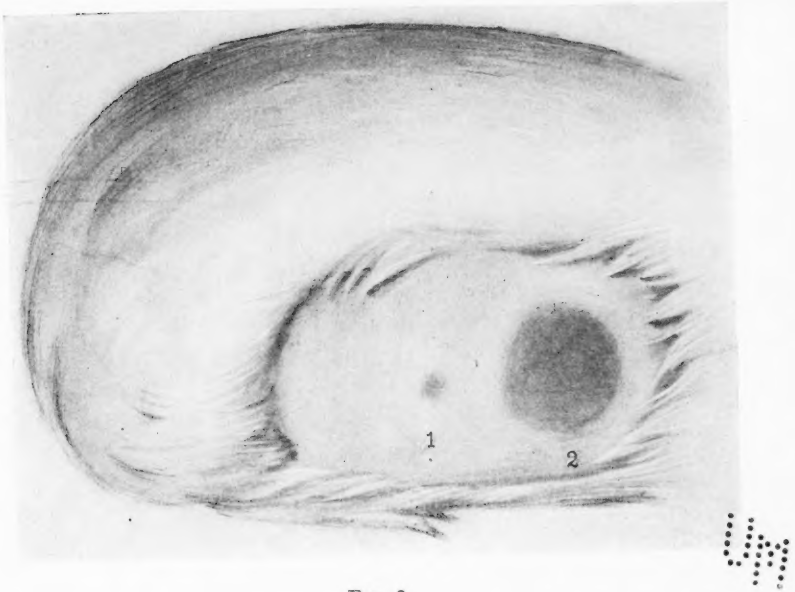

FIG. 2.

(Zinsser and Parker: Bacterial hypersusceptibility. II.)